BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA;

Predictive and Prognostic Biomarkers

Fiebo ten Kate

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BARRETT OESOFAGUS EN OESOFAGUS ADENOCARCINOOM;
Predictieve en Prognostische Biomarkers

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Voor mijn ouders

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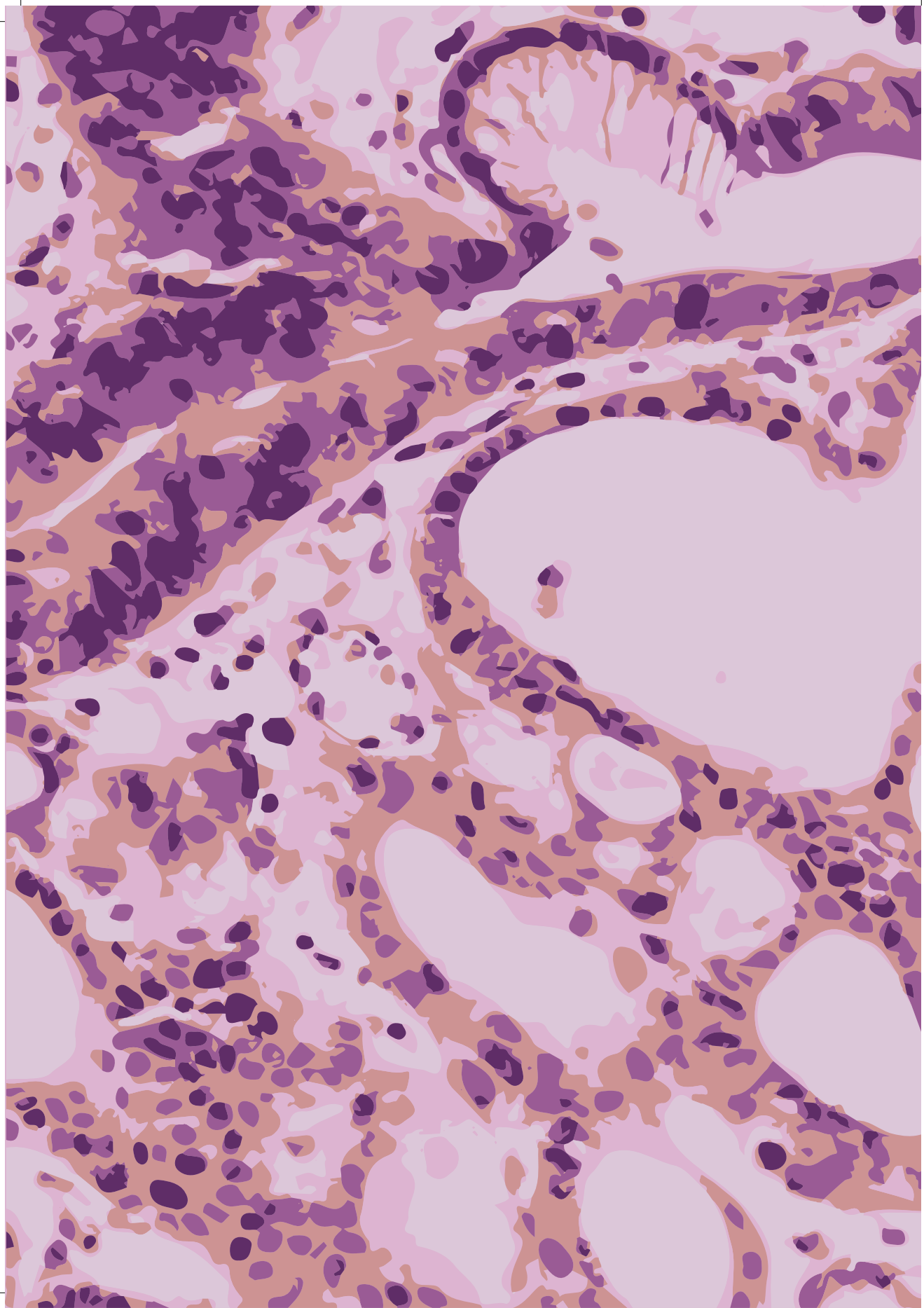
ABBREVIATIONS

ACG	American College of Gastroenterology
AUC	Area Under the Curve
BE	Barrett's Esophagus
CRT	Chemoradiotherapy
CI	Confidence Interval
CNV	Copy Number Variation
DAB	DiAminoBenzidine
DFS	Disease Free Survival
EAC	Esophageal Adenocarcinoma
FFPE	Formalin-Fixed Paraffin-Embedded
GI	Gastro-Intestinal
HR	Hazard Ratio
HGD	High Grade Dysplasia
HRP-ABC	HorseRadish Peroxidase Avidin-Biotin Complex
IHC	ImmunoHistoChemistry
IQR	InterQuartile Range
LGD	Low Grade Dysplasia
nCRT	Neoadjuvant ChemoRadioTherapy
NDBE	Non-Dysplastic Barrett's Esophagus
NPV	Negative Predictive Value
OS	Overall Survival
OR	Odds Ratio
PPV	Positive Predictive Value
ROC	Receiver Operating Characteristics
RR	Relative Risk
SNV	Single Nucleotide Variations
TMA	Tissue Micro Array
TNM	Tumor Nodes Metastasis

PART I

Introduction



CHAPTER 1

General introduction

GENERAL INTRODUCTION

Barrett's esophagus

Barrett esophagus's (BE) is the condition in which the normal multilayered squamous epithelium is replaced by a single row of columnar epithelium. In 1906, Wilder Tileston first mentioned the presence of metaplasia in the distal esophagus [1]. However, the recognition of chronic reflux disease in connection to the epithelial damage and development of columnar epithelium came to light only after the paper "Chronic peptic ulcer of the esophagus and esophagitis" published in 1950 by the British surgeon Norman Barrett [2]. Later on, correlation between BE and esophageal adenocarcinoma (EAC) was established, and increasing attention was placed on the diagnosis of BE. In the last decades, the incidence of BE is steadily rising in the Western world ranging from 1.6 to 7.8% of the general population [3-6]. Common predisposing factors for BE are white race, male gender, hiatus hernia, increased body mass index and increased abdominal fat, smoking and EAC in the first degree family members [3]. Chronic gastric-esophageal reflux disease (GERD) is the mayor risk factor for development of BE [7,8]. Another co-factor might be the world-wide decreasing incidence of Helicobacter pylori. A meta-analysis based on 15 observational studies showed a decreased risk for the development of EAC by more than 40% in patients with Helicobacter pylori infection [9].

Histological aspects of Barrett's esophagus and cell of origin

Since the first description of BE, there is a continuous discussion about the appropriate histological classification. In 1976, three different histological types were described. This included cardia type columnar epithelium with gastric features, fundus type with presence of parietal and chief cells, as well as intestinal type metaplasia [10]. Presently, it is recognized that BE is a complex multiclonal epithelium with mixed gastric and intestinal differentiation [11-13]. Since intestinal metaplasia is presumed to correlate with an increased risk of progression to EAC, Dutch guidelines recommend that diagnosis of BE is reserved for biopsies of endoscopically suspicious mucosa in which intestinal metaplasia is found on histology [14]. Accurate endoscopic and pathological correlation is important, since intestinal metaplasia might also be found in up to 30% of the normal gastro-esophageal transition zone [15].

It is not known where the metaplastic epithelium of BE originates from, but there are several hypotheses concerning the cell of origin. First of all, it has been postulated that squamous epithelium undergoes a direct metaplastic change [16,17]. Others suggested that the metaplastic epithelium originates from the subepithelial glands from the submucosa [18] or that gastric epithelium with stem like capacities migrates upward to the esophagus and colonize the damaged esophagus [19]. Another possible explanation is the persistence

of embryonic cells in the adult esophagus [20] and migration of stem cells from the bone marrow upon esophageal injury [21,22]. Lastly, a transitional zone within the gastro-esophageal junction could be the origin of the BE stem cells [23].

Histological and molecular progression in Barrett's esophagus

Patients with BE have an increased risk of developing EAC. This cancer develops through a step wise progression of BE to low grade dysplasia (LGD) and high grade dysplasia (HGD) (Figure 1). The histological criteria of LGD and HGD are poorly defined. In general, LGD shows a relatively intact glandular architecture in which adenomatous cytonuclear changes of the epithelium are present, including nuclear elongation, enlargement and hyperchromasia. The epithelium of LGD might show mild pleomorphism, mucin depletion, mild loss of polarity, nuclear crowding and nuclear (pseudo)-stratification. Furthermore, a clonal step, a sudden change from normal epithelium into epithelium with nuclear stratification, can be acknowledged.

The difference between LGD and HGD is largely based on a more complex architectural pattern, consisting of papillary or villous changes with branching crypts, complex budding in crypts or back-to-back crypts. The neoplastic cells of HGD show more pronounced cytological abnormalities compared to LGD. A non-adenomatous type of HGD is recognized in which profound nuclear abnormalities are noticed in the absence of nuclear stratification.

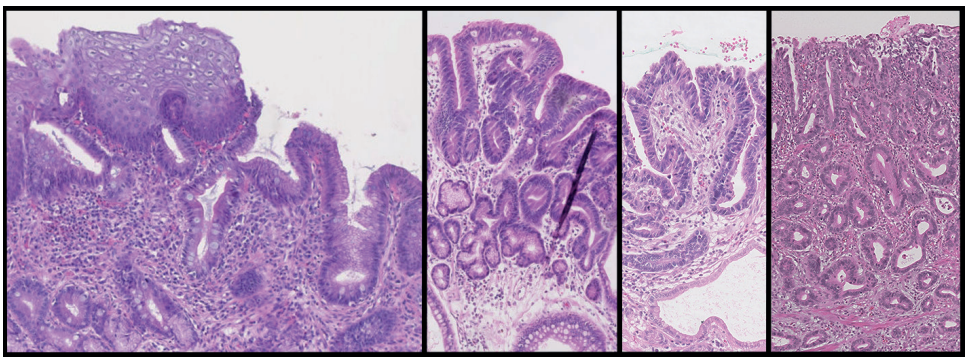


FIGURE 1: metaplastic change of normal squamous epithelium (far left) to esophageal adenocarcinoma (EAC) (far right) through BE without dysplasia, low grade dysplasia and high grade dysplasia.

The BE epithelium, even without dysplastic changes, shows highly polymorphic genetic landscape with multiple clones and extensive mutational load. Up to 6.7 single-nucleotide variants (SNV/Mb) are found in BE, which it is more extensive than in multiple myeloma

(1.1 SNV/Mb), hepatocellular carcinoma (3.7 SNV/Mb) and colorectal adenocarcinoma (5.9 SNV/Mb) [24]. In dysplasia, driver mutations are most commonly found in genes important for chromatin remodeling, such as *ARID1A* and *SMARCA* and tumor suppressor genes *TP53* and *SMAD4* [24-27]. Chromosomal instability of the BE steadily increases during malignant progression and extensive DNA damage with chromothripsis is found in a third of cases [28,29]. Two different pathways of malignant progression are proposed by Stachler *et al:* 1) starting with an early *TP53* mutation, followed by genome doubling and extensive genomic instability, and 2) starting with gradual loss of various tumor suppressor genes ending in a *TP53* mutation after which genomic instability arises (Figure 2) [26].

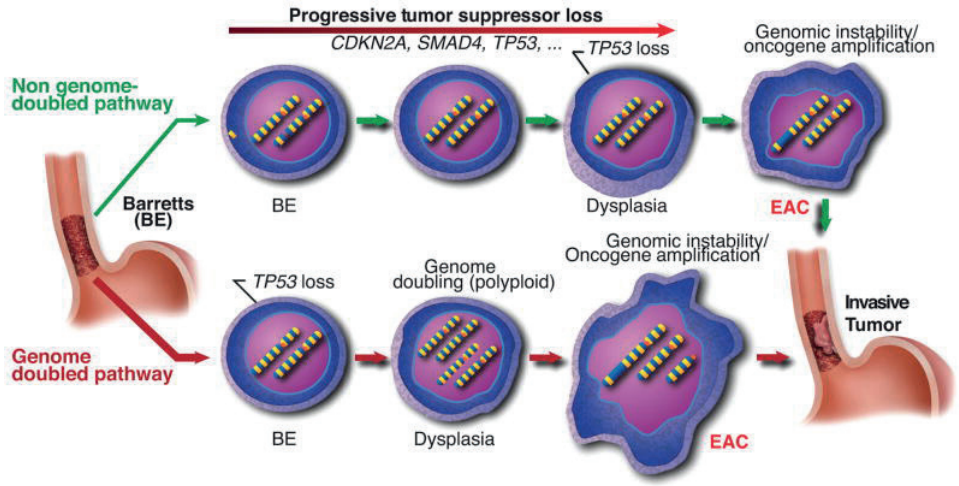


FIGURE 2: representation of the two different molecular pathways for progression of BE to EAC, as postulated by Stachler et al [26].

Follow-up and treatment of BE

Patients with BE have an increased risk of progression to EAC, compared to the general population. In three European population based studies the incidence of progression in NDBE was 0.12% - 0.43% [30-32], although in earlier published meta-analysis the calculated incidence was higher (0.41%-0.63%), probably because smaller studies with shorter follow-up data of selected groups of patients were included [33,34]. Since advanced EAC has a poor survival, patients with BE are offered endoscopic follow-up to detect progression at an early stage when EAC is still curable [35-38]. In a recent Dutch guideline a follow-up protocol is suggested based on the length of the BE segment [14]. According to this protocol, patient

with a BE segment of less than 1 cm do not require follow-up while in a BE segment of 1-3 cm or 3-10 cm endoscopic follow up should be five and three years respectively. If the BE segment is longer than 10 cm, the patient should be referred to an expertise center.
According to the Dutch guidelines, patients with LGD, confirmed by an independent expert pathologist, should be also referred to a clinical center with expertise. The follow-up of these patients is intensified, with an endoscopy after six months [14]. In patients with persistent LGD, ablative therapy of the Barrett segment can be considered. In case of HGD or early EAC patients extensive endoscopic work-up is necessary, followed by curative endoscopic resection of al visible lesions. In patients with EAC and high chance of nodal metastasis, radical surgery supplemented with chemotherapy and radiotherapy is indicated.
The frequency of endoscopic follow-up and subsequent treatment is mainly based on the pathological diagnosis. However, pathologic diagnosis of BE-related lesions, especially LGD, is problematic. Poor interobserver agreement for LGD has been frequently stated in the literature, with kappa value ranging from 0.11 to 0.35 [39-42], which can be interpreted as poor to fair agreement. This was confirmed in a recent work involving well-known expert gastro-intestinal (GI) pathologists from Europe and the US, again showing poor agreement for LGD [43]. Related to this, the progression rate of LGD to HGD or EAC is highly variable between the studies (< 1% and 13%) [30-32,44]. A meta-analysis has shown that studies in which LGD is more prevalent the chance of progression is lower [44].
Because of these observations, the predictive value of LGD was generally considered to be very low. However, during recent years multiple studies, mostly from Dutch expert centers, have shown improved prediction capacity when LGD was confirmed by a panel of expert pathologists (annual progression rate of 27%) [45], and the chance of progression increased with every additional pathologist confirming the LGD diagnosis [46].
Although the diagnostic criteria, as mention above, seem quite straight forward they are open for interpretation. Furthermore, it is not clear which criteria are required for the diagnosis of LGD.

Biomarkers to predict progression in Barrett's esophagus

Selected biomarkers (indicators of presence or absence of a pathologic state or process, in this case BE), could be used to improve the predictive value of histological diagnosis, in other words an indicator which patient will progress to EAC and which patient will not show progression. Multiple biomarkers have been tested earlier [47,48]. The most used biomarker to date is P53, the well-known "guardian of the genome" [49], encoded on *TP53* which is one of the most studied genes in human cancer. *TP53* is mutated in up to 70% of the EAC as found by whole genome and exome sequencing studies [24-27]. Expression of P53 was related to the outcome previously by us and others [50-56]. Normal immunohistochemical staining of P53 is defined as a faint nuclear staining while aberrant expression includes strong nuclear

expression (called overexpression) or complete loss of expression. Aberrant expression of P53 is correlated to an increased chance of progression with an odds ratio (OR) of seven in a recent meta-analysis [47]. But not only is P53 predictive of progression it also improves the interobserver agreement for LGD diagnosis [53,57].

Another promising marker related to proliferation is Cyclin A. This protein controls progression by activation of cyclin-dependent kinase enzymes, and is expressed in the S and G2 phase of the cell cycle. The results on Cyclin A as predictive marker in BE are conflicting. Overexpression of this protein in BE has been inconsistently correlated with progression to EAC, but reactive epithelium in the background of inflammation may also show increased mitotic activity and Cyclin A expression.

Another promising biomarker is SOX2, a transcription factor which is essential to remain the pluripotent capacities of stem cells [58]. SOX2 has been shown to be expressed in squamous epithelium of the esophagus as well as foveolar epithelium of the stomach [59-61]. Although SOX2 has been introduced as an oncogene in squamous cell carcinoma, its functions are highly cell specific. In gastric tissue, SOX2 is downregulated during progression from metaplastic epithelium into gastric carcinoma and may inhibit proliferation and invasiveness of the tumor cells [60-62].

P53 is currently the only accepted immunohistochemical marker in clinical practice according to the Dutch and British guidelines [14,35]. Other biomarkers are presently not recommended due to insufficient knowledge of their predictive value.

Treatment of esophageal adenocarcinoma in early and advanced stage

Neoplastic progression of NDBE can lead to the development of EAC, which is a highly aggressive neoplasm with poor prognosis in the advanced stages. Radical esophagectomy, for decades the only curative treatment of EAC, is a major operation with a high mortality and morbidity [63]. In the nineties of last century endoscopic mucosal resection was shown to be a good alternative for the treatment of early invasive EAC. The prerequisite is that the risk of lymph node metastasis (LNM) have to outweigh the risk of radical surgery [35,64,65]. The risk of LNM in tumors confined to the mucosa are considered to be very low while LNM risk in EAC invading in the submucosa is higher, ranging 3-44%. The LNM risk is difficult to predict in the individual patient but in generally it depends on tumor characteristics such as tumor grade, depth of invasion and lympho-vascular invasion (LVI) [66-69]. Well to moderately differentiated EAC with superficial invasion of the submucosa (submucosal invasion of less than 500 µm) and without LVI has a low chance of LNM (3-6%). Additional surgical treatment could be speared in these patients, since radical surgery has a 5% mortality and high morbidity rate of around 50% [70-72].

Treatment of an advanced EAC has also undergone profound changes during the last decades. In short, until the early eighties of last century patients with a more advanced

EAC were treated by radical gastro-esophagectomy as single treatment modality. Over 80% of the patients developed, in these days, local or systemic recurrence, usually within six to twelve months [67,73,74]. Triggered by these poor survival rates, interest developed for the use of multimodality therapy, consisting of neoadjuvant chemotherapy, radiotherapy or a combination of both. Several randomized controlled trails have been performed comparing surgery alone and combined treatment with neoadjuvant chemo(radio)therapy prior to resections. The regimes included either cisplatin and combined chemotherapeutics (cisplatin and fluorouracil or cisplatin, fluorouracil and epirubicin) [73]. These studies from Japan, France and the United Kingdom discovered survival benefit for those patients who received neoadjuvant treatment [75-77]. The Dutch multicenter CROSS-trail (ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study) was initiated in 2004, which compared surgery alone with surgery plus chemotherapy consisting of carboplatin and paclitaxel, and radiotherapy, consisting of 23 fractions of 1.8 Gy. This randomized controlled trial showed a treatment benefit for patients treated with neo-adjuvant chemoradiotherapy (CRT) and surgery (hazard ratio (HR) 0.657) [78]. In the resection specimens of patients treated by CROSS in the CRT arm, 23% showed a complete response, defined as a ypT0N0. This fact has led to further developments in the field of EAC surgery, including multicenter Pre-SANO (surgery as needed in oesophageal cancer) trial [79] showing high diagnostic accuracy for assessment of residual disease after neoadjuvant treatment and subsequent start of the SANO trial [80].

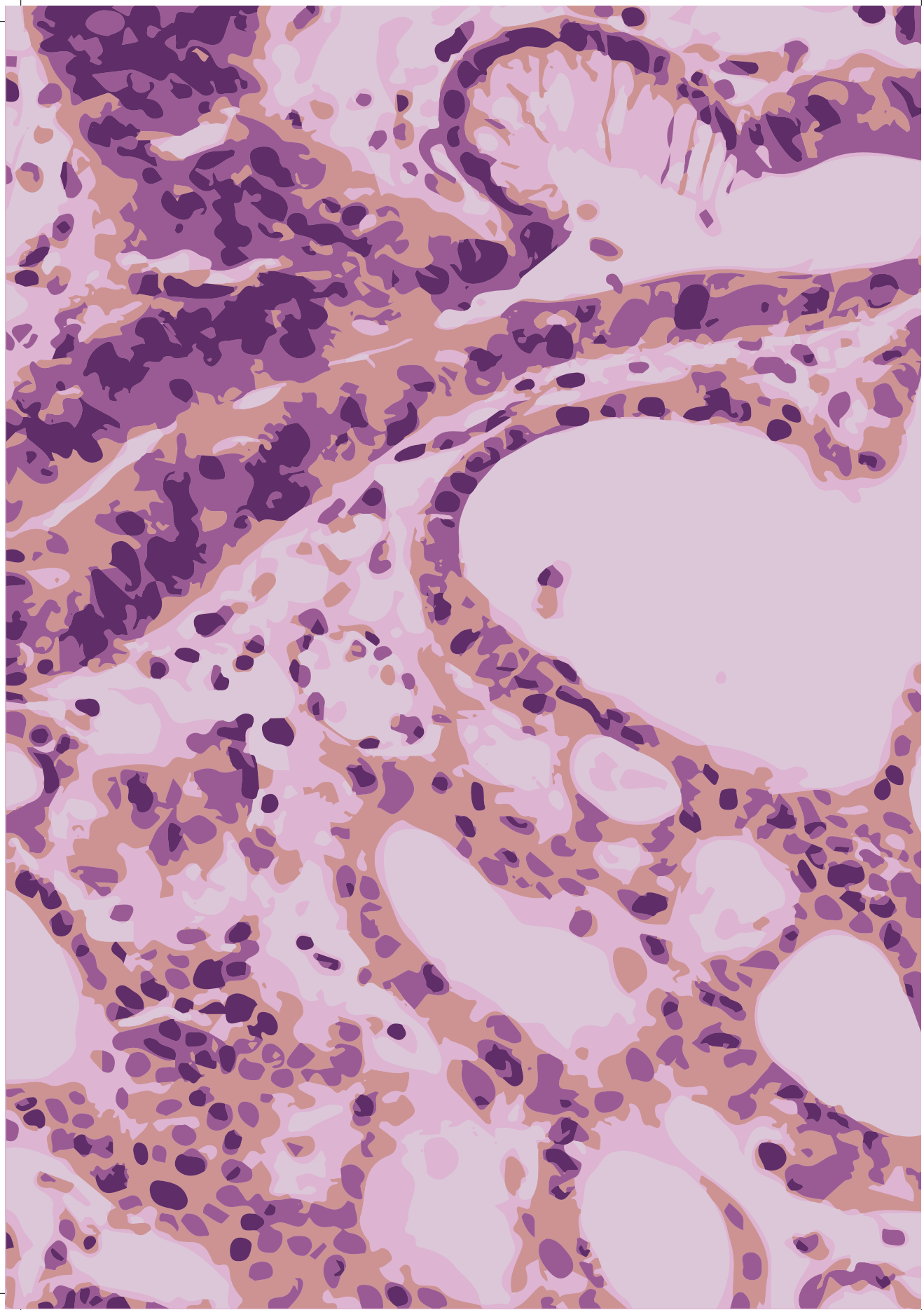
Biomarkers for the prediction of lymph node metastasis and prognostication in patients with EAC

The prognosis in patient with EAC is dependent on various clinical and histological parameters. As the matter of fact, it is difficult to predict if the patient will show rapid progression of the disease or will have a more favorable outcome. Rapid progression is mainly caused by the development of distant metastasis or local recurrence. Introduction of neoadjuvant treatment has led to an improved prognosis in general, but the individual response is highly variable. Additional biomarkers in early and advanced EAC could improve survival prediction and treatment. In pT1b EAC, being EAC invading into but not beyond the submucosa of the esophagus, the prediction of LNM is currently based on histopathologic criteria, namely tumor differentiation, infiltration depth into the submucosa and lympho-vascular invasion. No other biomarkers are used so far in early EAC to predict LNM.

In advanced EAC, TNM-classification is the only clinically used system for the prognostication of patients [75]. With the use of this classification, based on the depth of tumor invasion and the number of LNM and distant metastasis, an indication of prognosis for the individual patient can be given [81], although further specification for the individual patient is needed.

In the quest to improve the prognostication of individual patients several biomarkers have been tested [82-84]. Since squamous cell carcinoma (SCC) of the esophagus is worldwide

the most prevalent carcinoma of the esophagus, most studies include only SCC or a combination of SCC and EAC. Studies focusing on EAC showed that COX2, EGFR, HER2, KI67 and P53 could be of value as predictive biomarkers in subset of EAC [82,83]. However, the results of the previous studies are difficult to interpret because of the various treatment regiments of the patients included. Also, none of the studies could show predictive value of these biomarkers for detection of LNM.



CHAPTER 2

Outline of the thesis

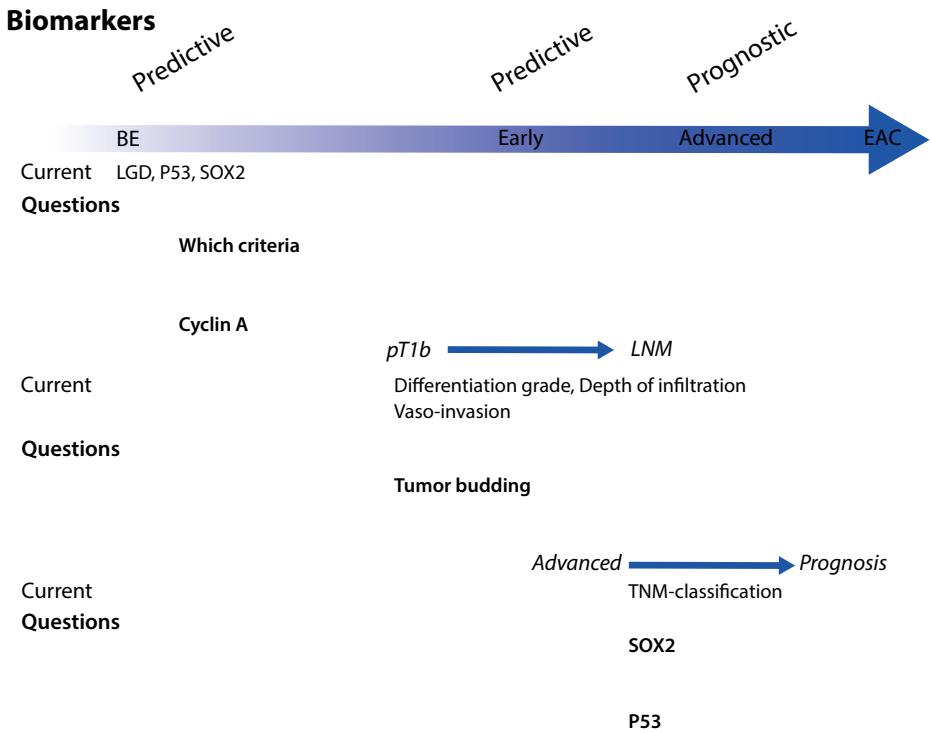


FIGURE 1: Schematic overview of this thesis. In Part II the predictive value of biomarkers for the progression of Barrett's esophagus (BE) to esophageal adenocarcinoma (EAC) is assessed, especially the specific histological criteria for low grade dysplasia (LGD) and the additive role of Cyclin A to the know biomarkers SOX2 and P53. In Part III of this thesis the predictive value of tumor budding in early EAC is assessed, in addition to the currently used histological criteria (differentiation grade, depth of invasion and vaso-invasive growth), and the prognostic value of P53 and SOX2 in advanced EAC besides the presently used tumor node metastasis (TNM) system.

OUTLINE OF THE THESIS

It is important to improve the risk stratification of patients with BE as well as the prognostication in patients with established EAC. With this thesis we aimed to evaluate if optimal histological evaluation and use of biomarkers can help to achieve these goals.

Part II

In **chapter 3** and **chapter 4** two studies are presented aiming to improve prediction of progression in patients with BE. In **chapter 3**, the histological criteria for LGD are evaluated in two independent groups of patients with BE to select those criteria with the highest reproducibility between observers and highest value to predict progression to HGD or EAC. In **chapter 4** the value of Cyclin A as predictive biomarker was evaluated in a large cohort of patients with BE and compared to the predictive value of other biomarkers such as P53, SOX2 and AMACR.

Part III

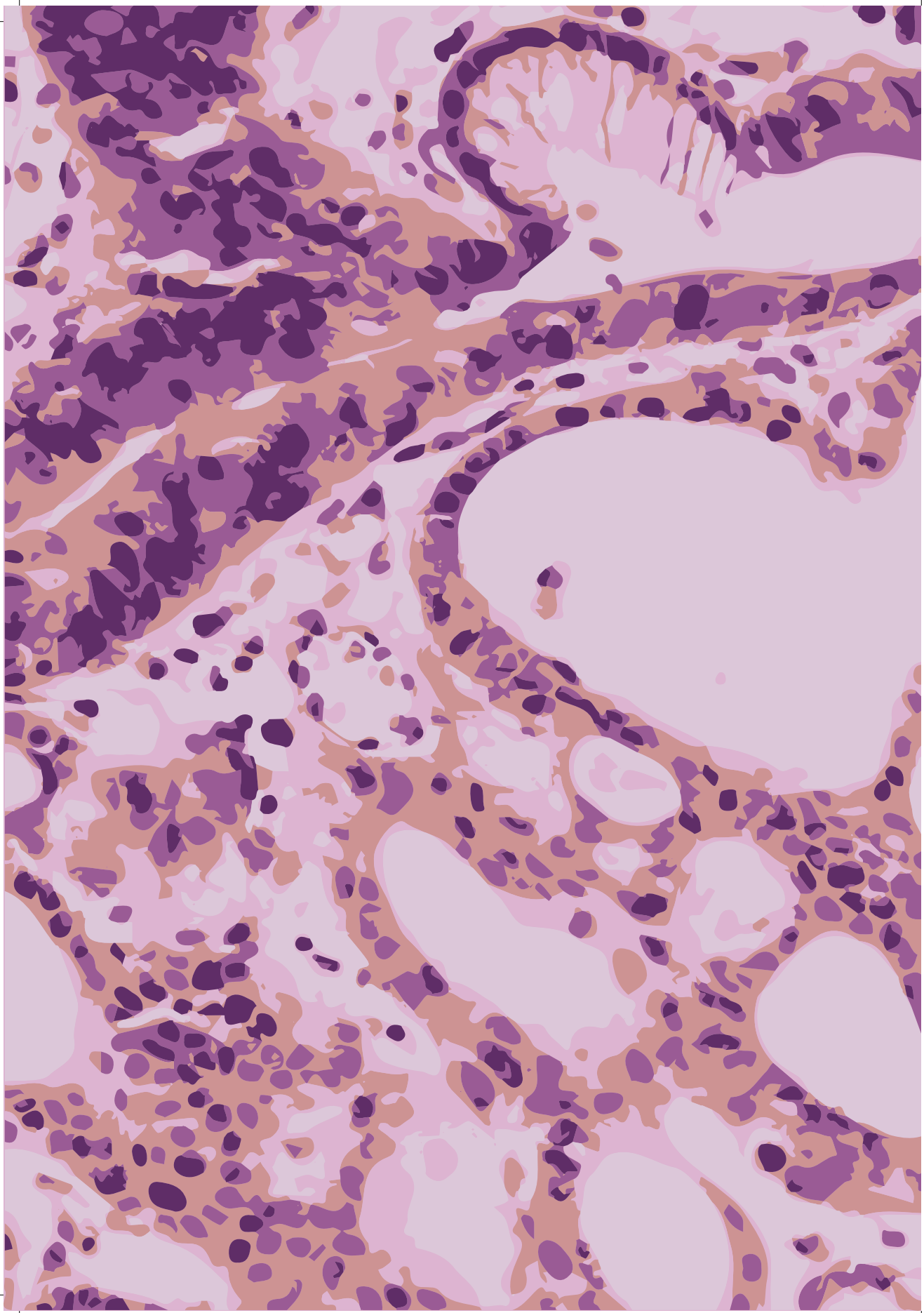
In **chapters 5-7** predictive markers in established EAC are evaluated. In **chapter 5** tumor budding is studied in early (pT1b) EAC and validated in additional pT1b EAC cohort. To improve the prognostication of patients with advanced EAC the value of the immunohistochemical markers, SOX2 and P53 are tested in **chapters 6** and **7**. Next to the immunohistochemical evaluation of resection specimens, molecular analysis including DNA sequencing and high-throughput methylation analysis are performed to reveal underlying genetic changes.

Part IV

Finally, in **chapter 8 and 9** the results of this thesis are discussed and summarized.

PART II

Surveillance of Barrett's esophagus



CHAPTER 3

Improved progression prediction in Barrett's esophagus with low grade dysplasia using specific histological criteria

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ABSTRACT

Introduction: Risk stratification of patients with Barrett's esophagus (BE) is based on diagnosis of low grade dysplasia (LGD). LGD has a poor interobserver agreement and a limited value for prediction of progression to high grade dysplasia (HGD) or esophageal adenocarcinoma (EAC). Specific reproducible histological criteria may improve predictive value of LGD.

Methods: Four GI-pathologists examined 12 histological criteria associated with LGD in 84 BE patients with LGD (15 progressors and 69 non-progressors). The criteria with at least a moderate (kappa 0.4-0.6) interobserver agreement were validated in an independent cohort of 98 BE patients with LGD (30 progressors and 68 non-progressors). Hazard Ratios (HR) were calculated by Cox proportional hazard regression analysis using time-dependent covariates correcting for multiple endoscopies during follow-up.

Results: Agreement was moderate or good for four criteria, i.e., loss of maturation, mucin depletion, nuclear enlargement and increase of mitosis. Combination of the criteria differentiated high- and low risk group within the patients with LGD diagnosis ($p<0.001$). When two or more criteria were present a significantly higher progression rate to HGD or EAC was observed (discovery set: HR 5.47, 95% CI 1.81-17, $p=0.002$; validation set: HR 3.52, 95% CI 1.56-7.97, $p=0.003$). Implementation of P53 immunohistochemistry and histological criteria optimized prediction of progression (area under the curve 0.768 (95% CI 0.656-0.881)).

Conclusion: We identified and validated a clinically applicable panel of four histological criteria, segregating BE patients with LGD diagnosis into defined prognostic groups. This histological panel can be used to improve clinical decision making, although additional studies are warranted.

INTRODUCTION

The major risk factor for esophageal adenocarcinoma (EAC) is Barrett's esophagus (BE), a condition in which squamous epithelium of the distal esophagus is replaced by columnar epithelium with gastric and colonic differentiation. The EAC pathogenesis is suggested to be a gradual process with intermediate stages of low grade dysplasia (LGD) and high grade dysplasia (HGD) [35,85]. The overall incidence of progression from BE to HGD or EAC is low (0.13-0.15% per year), as demonstrated by multiple BE cohort studies from different countries [31,32]. As a result, the rationale for BE surveillance as well as optimal approach for BE patients remains debated [86]. Endoscopic surveillance programs offer the opportunity for early detection and treatment of relevant neoplastic lesions in order to prevent development of advanced cancers [31,32]. Diagnosis of LGD in biopsies taken during Barrett surveillance is an important prognostic indicator for progression and the reason to intensify surveillance interval [8,35,36,85]. Alternatively, radiofrequency ablation might be indicated [87]. Current guidelines recommend endoscopic eradication therapy in patients with confirmed and persistent LGD with the goal of achieving complete eradication of intestinal metaplasia [87,88].

In patients with LGD, major differences in rates of progression to HGD/EAC are reported in previous studies, varying from <1% to up to 13.4% per patient-year [39,45,86,89-91]. The differences in progression rate might reflect difficulties in discriminating true neoplasia from BE with reactive changes. Recent studies indicate that the predictive value of LGD diagnosis increases after expert review confirmation [45,90,92]. Based on this observation, LGD should be confirmed by a second pathologist with experience in gastro-intestinal- and especially in BE-pathology [35,85,88]. However, overall interobserver variation for the diagnosis of LGD remains significant even amongst expert pathologists, with kappa values reported to be poor in most studies [40-42]. Adoption of standards for LGD diagnosis would increase agreement, but the descriptive histological criteria for LGD are not sufficiently harmonized yet [40,43]. Therefore, the aim of the present study was to challenge the histological criteria for LGD for their reproducibility and capacity to predict progression. We propose that a defined histological criteria panel could improve prediction of progression in BE patients with LGD and thereby improves risk stratification in BE patients.

METHODS

Setting and patients population

The study aimed to improve predictive value of LGD. Therefore, we examined the reproducibility of selected histological criteria and tested their power to predict progression in patients with a Barrett's esophagus, which was defined by development of HGD or EAC. Two independent cohorts of BE patients were identified retrospectively. The characteristics of both study populations are shown in Figure 1A.

The discovery set consisted of patients under endoscopic surveillance for BE at Erasmus Medical Center (EMC) (Rotterdam, the Netherlands), with at least one pathological record of LGD during follow up (LGD diagnosis was made between 2003-2014). Patients with LGD or HGD in their medical history had at least one year of follow-up before being eligible for inclusion in this study.

The validation set consisted of patients with BE included in the ProBar study [93], with LGD diagnosis made on follow up. The study protocol has been described before [51,93,94]. In short, the ProBar study is a prospective study comprised of more than 700 patients with known or newly diagnosed BE. The endoscopic diagnosis of BE was histologically confirmed by the presence of intestinal metaplasia. Patients with HGD or EAC on index endoscopy or a history of HGD or EAC were excluded from the ProBar study and were not encountered for the validation cohort. The ProBar patients were followed until they developed HGD or EAC, at which point they were treated and excluded from further follow-up. Of this cohort all patients with LGD and progression to HGD or EAC during follow-up were selected and matched to patients with LGD during follow-up, but without progression to HGD or EAC in ratio of 1:2.

All biopsies of the patients from Erasmus MC and the ProBar cohort were independently reviewed by two expert pathologists who confirmed the presence of LGD diagnosis before evaluating the criteria. If these pathologist were discordant on the grade of dysplasia a third expert pathologist reviewed the case. Only biopsies with a consensus diagnosis of LGD were included in this study. The presence of HGD or EAC in progressors was also reviewed and confirmed by four expert pathologists (MD, KB, FK and FJWtK), all actively participating in national BE studies, having extensive experience in the assessment of BE pathology [45,51,95]. Data analysis was performed based on histological diagnosis on follow-up.

Endoscopic follow-up

Clinical follow up of all included patients was performed according to the guidelines of the American College of Gastroenterology, with a standardized endoscopy protocol, performed by experienced gastroenterologists [38]. Upper endoscopy biopsies were taken according to

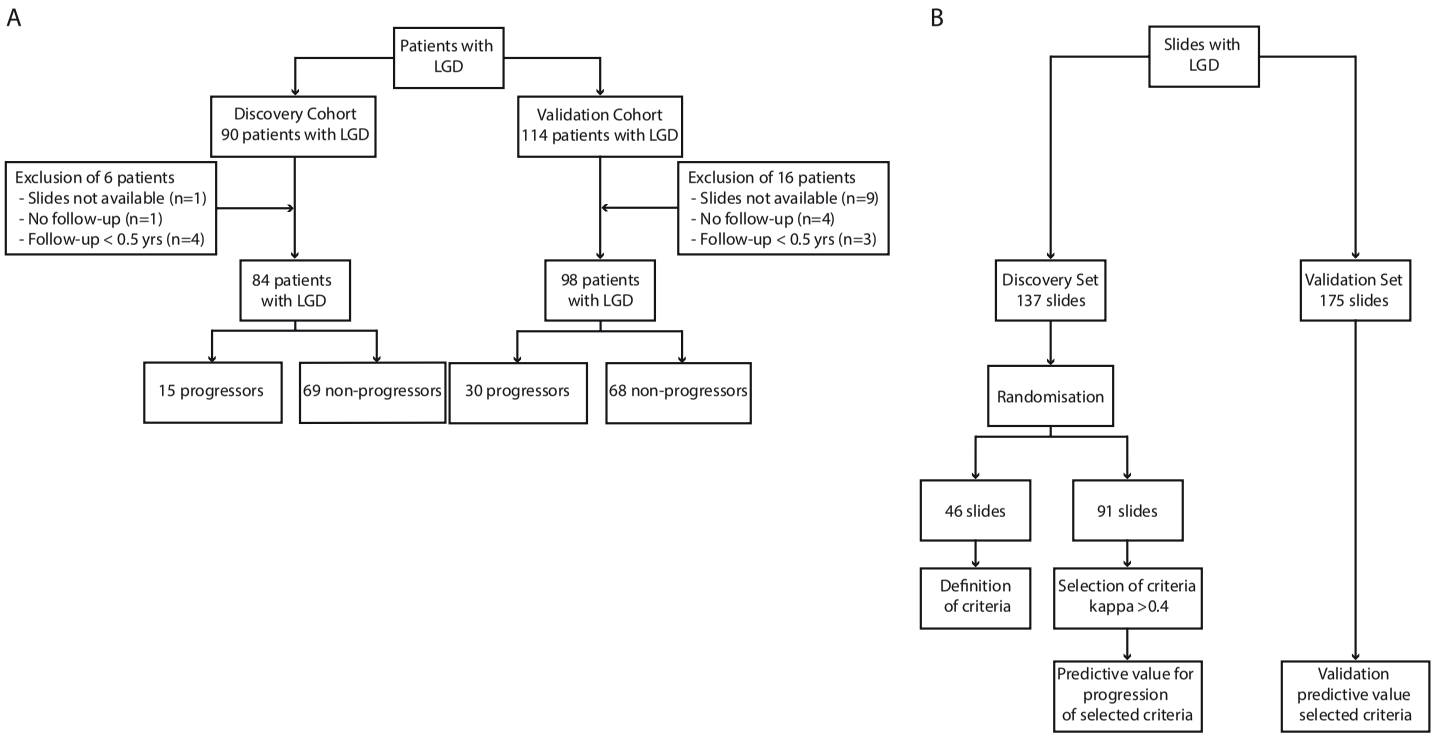

FIGURE 1: Flow chart of patients in this study (A) and study design (B). All slides of the discovery cohort were randomised and were assigned in a consecutive number from one to 137. The first 46 slides were used as learning set to define the criteria, the rest of the slides were used to calculate the interobserver correlation and the correlation to time to neoplastic progression. LGD: low grade dysplasia. Progressors were defined as patients who developed high grade dysplasia or esophageal adenocarcinoma at follow-up. Non-progressors were defined as patients without neoplastic progression during follow-up.

the Seattle protocol [96]. Duration of follow-up was calculated for each patient from the date of LGD endoscopy to the most recent endoscopic procedure with biopsies or the date of endoscopy in which HGD or EAC was diagnosed.

Study design

Several histological criteria for LGD are mentioned in the guidelines of the British Society of Gastroenterology [35]: loss of surface maturation, clonal step (sharp demarcation between non-dysplastic epithelium and normal/reactive epithelium), loss of polarity, mucin depletion, stratification of nuclei, nuclear form and nuclear features (enlargement, pleomorphism, hyperchromasia, prominent nucleolus), as well as increase in apoptosis and mitosis. To refine these histological criteria, all four participating GI pathologist discussed each of the individual criterion in a consensus meeting and specific definitions for each of the criteria were documented. Therefore, 17 H&E slides of patients with LGD diagnosis and progression on follow-up and 29 slides of patients without progression were used from the discovery set. Thereafter, all refined criteria were applied by each of the four pathologists on the remaining slides of the discovery set (20 H&E slides of 11 progressors and 71 slides of 57 non-progressors). The most reproducible histological criteria defined by kappa value > 0.4 were selected for further statistical analysis and correlation with clinical data.

Next, the criteria were validated in patients from ProBar-study, using 58 H&E slides of 30 patients showing progression and 117 slides of 68 patients without progression. The H&E slides were individually reviewed by two pathologists (FK and MD). If discordant on one of the selected criteria, a third pathologist (KB) reviewed the slide for all four histological criteria.

All samples of patients in the discovery cohort and validation cohort were reviewed for the presence of histological criteria for LGD. The pathologists involved were blinded to the diagnosis of each other as well the clinical and histological follow-up results. The consensus was defined as such when two or more pathologists agreed on presence or absence of each criterion. The flow diagram of the study design is shown in Figure 1B. In case of multiple biopsies with LGD during follow up in one patient, the results from the index biopsy were used for the statistical analysis (see below).

Ethics

The study was approved by the Institutional Review Board of the EMC (code MEC-2016-042) and local medical ethical committees of all participating hospitals. Based on the opt-out registry, used in the EMC to document the objection of patients to use excess tissue materials for scientific research, none of the included patients had opposed.

Statistical analysis

Median and interquartile ranges (IQR) were calculated for continuous variables. Characteristics of progressors and non-progressors were compared using Mann-Whitney U-test for continuous variables and X^2 test for categorical variables. Biopsies were analyzed for interobserver agreement on all individual histological criteria, by using Fleiss kappa for the discovery set [97] and Cohens kappa for the validation set. Strength of agreement was categorized as follows: 0.00-0.20 = poor; 0.21-0.40= fair; 0.41-0.60 = moderate; 0.61-0.80 = good; and 0.81-1.00 = very good [98].

Cumulative risk for progression was calculated using Kaplan-Meier survival curves. The impact of pathological criteria on time until progression was quantified using Cox regression with time dependent covariates [77], frailty terms were included for discovery set to account for patients with multiple progressions [99]. In the validation set we performed Cox regression analysis with time-dependent covariates, no frailty terms were required as each patient had at most 1 progression. Multivariable Cox regression was corrected for patient age at endoscopy, length of the Barrett segment and the presence of esophagitis. The predictive value of the combination of criteria was calculated after the optimal cutoff was determined using a Receiver Operating Characteristic (ROC) Curve and Youdens-index.

Statistical calculations were performed using the statistical package for the social sciences (SPSS 20.0, IBM Corp., Armonk, New York, USA) and R version 3.2.1 (Vienna, Austria). Fleiss kappa was calculated using the irr package in R, Cox regression was performed using the survival package in R.

RESULTS

Patients and characteristics

In total 204 patients with BE were originally included in this study, 90 in the discovery and 114 in the validation set (Figure 1A). After exclusion for various reasons, 84 and 98 BE patients remained in discovery and validation set respectively. From 15 progressors in the discovery set, 11 had HGD in the past (treated by radiofrequency ablation and endomucosal resection), in contrast to none of the 30 progressors in the validation set who had no prior history of HGD or EAC.

TABLE 1: Demographics of all included Barrett's esophagus patients.

	Discovery set n=84	Validation set n=98	p-value
Age at biopsy, Median, years (IQR)	67.7 (57.9-74.0)	70.7 (62.9-75.6)	0.025§
Sex			
Male	69 (82.1%)	76 (77.6%)	0.443°
Female	15 (17.9%)	22 (22.4%)	
Smoking			
Yes	12 (14.3%)	11 (11.2%)	0.266°
No	57 (67.9%)	86 (87.8%)	
Not available	15 (17.9%)	1 (1.0%)	
Use of Alcohol			
Yes	52 (61.9%)	72 (73.5%)	0.783°
No	17 (20.2%)	26 (26.5%)	
Not available	15 (17.9%)	0 (0.0%)	
Esophagitis during follow-up			
Yes	4 (4.8%)	88 (89.8%)	0.264*
No	80 (95.2%)	10 (10.2%)	
Length of BE, Median (IQR)	5.0 (3.0-7.0)	5.0 (3.0-7.0)	0.994§
Follow-up, Median, Years (IQR)	7.5 (3.5-9.1)	5.3 (2.8-8.4)	0.191§
Endoscopies, Median number (IQR)	5.5 (4.0-6.75)	6.0 (4.0-7.0)	0.123§
Number of biopsies from individual patient, Median number (IQR)	1.0 (1.0-2.0)	1.0 (1.0-2.0)	0.967

BE: Barrett's esophagus; IQR: Inter Quartile Range; ° Pearson Chi-square test; * Fisher's exact test; § Mann-Whitney U test

Patient characteristics of the finally included cases in both data sets are given in Table 1. No statistical differences between both cohorts were found concerning sex, BE length, time of follow-up or number of endoscopies performed. The patients of the discovery set were significantly younger, with a median age of respectively 67.7 years compared to 70.7 years in the validation set (p=0.025). The patient characteristics specified for progressors versus non-progressors are given in supplemental Table 1.

Histological criteria for LGD and prediction of progression in the discovery set

Four pathologists scored all H&E slides from the discovery set patients using the 12 histological criteria for LGD [35] which had been discussed and specified by the involved pathologists during a prior consensus meeting (supplemental Table 2). Eight criteria showed a poor to fair interobserver agreement (kappa -0.16 – 0.36) in the discovery set and were disregarded from further analysis (supplemental Table 2). The remaining four criteria, including loss of surface maturation (defined as no maturation of the epithelium seen on low power from the proliferation zone until the surface), mucin depletion (defined as almost total to total disappearance of mucus from the surface columnar cells on high power), nuclear enlargement (defined as a nuclear size at least 2x as large as nuclei of the normal not inflamed columnar epithelium) and increase of mitosis (defined as at least one mitosis at the epithelial surface or in the neck of the crypts, mitosis in the base of the crypt are disregarded), had a moderate agreement in the discovery set (kappa value of 0.55, 0.51, 0.41 and 0.48 respectively). The percentage of agreement for these criteria varied between 64.9% and 91.5% (supplemental Table 3). Histological examples of the four criteria are given in Figure 2. In the multivariable Cox regression analyses, corrected for gender, age, length of BE and esophagitis, all four parameters were significantly associated with neoplastic progression (Table 2, HR respectively: 5.93 (95% CI 2.02-17), 4.54 (95% CI 1.55-13), 4.23 (95% CI 1.28-14) and 7.27 (95% CI 2.46-21; see also supplemental Table 4 for univariable analysis). When combining these four criteria in a single panel, the most predictive cutoff for progression was calculated using a ROC-curve and corresponding Youden index (supplemental Figure 1 and supplemental Table 5). This panel was considered to be positive if two or more criteria were present. Differences in progression time were found depending on the number of criteria positive; 9.0 years (95% CI 8.2-9.8) for LGD with up to one criterion compared to 3.8 years (95% CI 3.0 - 4.7) for LGD with two or more criteria. The corresponding Kaplan Meier curve is depicted in Figure 3a. This shows a clear separation between patients with up to one criterion and more than two criteria, also if compared to the LGD diagnosis alone. During follow-up of maximal 10 years 9.9% of the patients with up to one criterion showed progression in comparison to 43.8% in biopsies with two or more criteria present (see supplemental Table 6). In a multivariable Cox regression analysis

patients with 2-4 criteria in their first biopsy with LGD showed a significantly higher risk of progression to HGD and EAC compared patients with up to one criteria (HR 5.47, 95% CI 1.81-17, p=0.002).

Validation of the histological criteria panel and individual contribution of the criteria for the prediction of progression

The interobserver agreement and predictive value of the criteria loss of surface maturation, mucin depletion, nuclear enlargement and increase of mitosis, were validated on the independent patient set. Two expert pathologists (MD and FK) evaluated 175 H&E slides of 98 patients followed prospectively in the ProBar-study. Thereby, a moderate or good interobserver agreement for all 4 criteria was found (kappa values: loss of maturation 0.61, mucin depletion 0.50, nuclear enlargement 0.47, increase of mitosis 0.46, combination of the criteria 0.61; see supplemental Table 2).

Panel consisting of these four distinct histological criteria segregated patients with LGD diagnosis into prognostic groups (p<0.001) (see Figure 3b for corresponding Kaplan Meier curve). When correlating with follow-up by multivariable Cox regression analysis, these criteria were significantly associated with neoplastic progression (HR respectively; 3.41 (95% CI 1.52-7.67), 2.76 (95% CI 1.28-5.96), 4.01 (95% CI 1.84-8.73) and 2.91 (95% CI 1.36-6.24)) (see Table 2, univariable analysis in supplemental Table 4). Patients with more than two criteria in their index LGD biopsy showed a significantly higher risk of progression to HGD or EAC compared to patients with up to one of the criteria (HR 3.52, 95% CI 1.56-7.97, p=0.003; see Table 2). Data on progression incidence per patient-year, as well as 2- and 5-year cumulative risk of progression are given in supplemental Table 6.

TABLE 2: Hazard ratios (HR) for individual histological criteria and combination of these criteria in a multivariable Cox regression analysis for the prediction of progression to high grade dysplasia or esophageal adenocarcinoma.

	HR in multivariable analysis					
	Discovery set			Validation set		
Histologic al criteria	HR	95% CI	P-value	HR	95% CI	P-value
Loss of surface maturation	5.93	2.02-17	0.001	3.41	1.52-7.67	0.003
Mucin depletion	4.54	1.55-13	0.006	2.76	1.28-5.96	0.010
Nuclear enlargement	4.23	1.28-14	0.018	4.01	1.84-8.73	<0.001
Increase in mitoses	7.27	2.46-21	<0.001	2.91	1.36-6.24	0.006
Combination of criteria (ref 0-1) 2-4 criteria present	5.47	1.81-17	0.002	3.52	1.56-7.97	0.003

Adjusted for gender, age, length of Barrett's esophagus and esophagitis. HR: Hazard ratio, CI: Confidence Interval

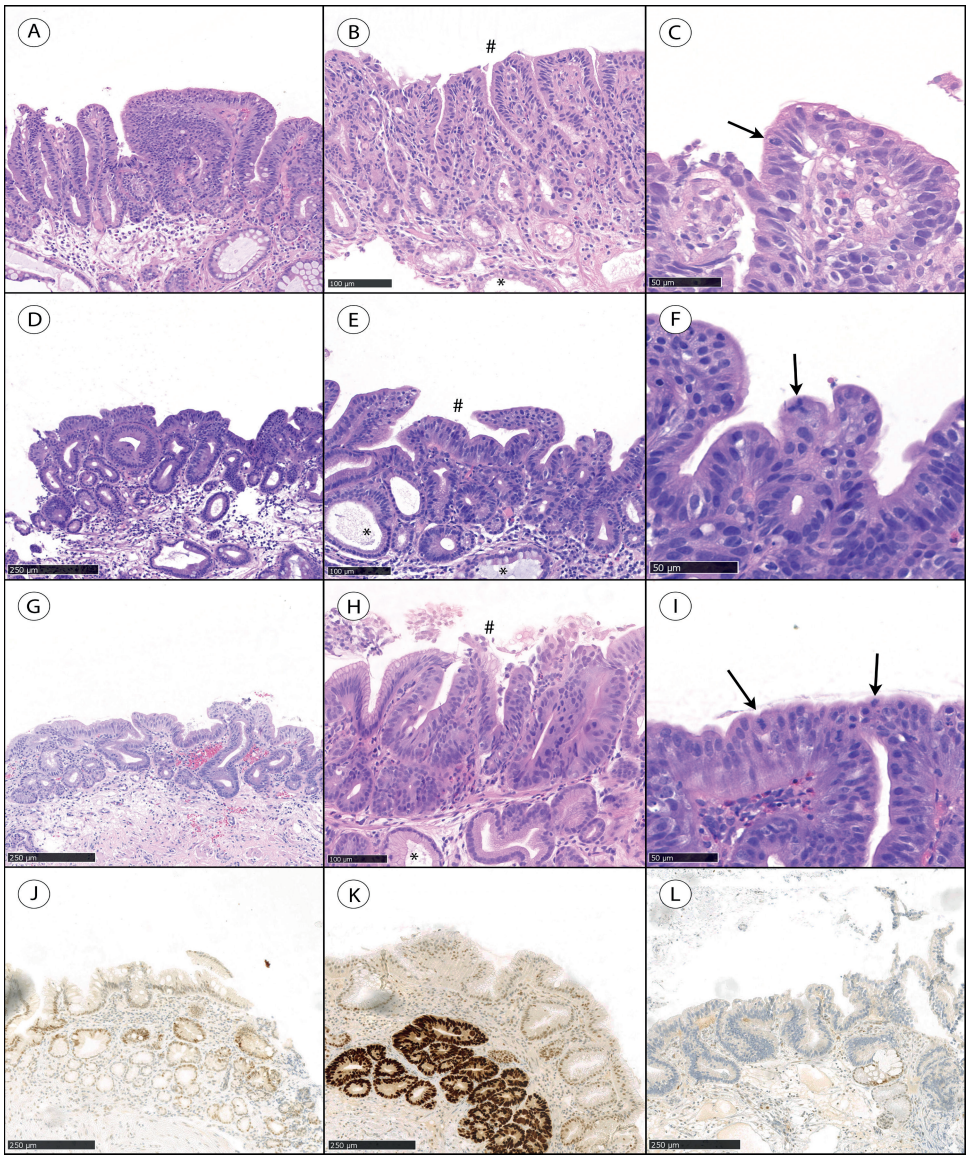

FIGURE 2: Examples of the histological criteria and of the expression of P53. A, D, G: loss of surface maturation, i.e. lack of normal epithelial maturation from the proliferation zone until the surface (all 100x magnification). B, E, H: mucin depletion, i.e. total or almost total disappearance of mucus from the surface columnar cells. Furthermore nuclear enlargement can be appreciated if the dysplastic cells (indicated by #) are compared to the normal epithelium (indicated by *) (all 200x magnification). C,F,I: increase in mitosis, indicated by arrows, present at the luminal side of the biopsy or in the neck of the crypt (all 400x magnification). J, K, L: example of P53 expression; J: normal expression of P53 with weak nuclear staining. K: overexpression of P53 with strong nuclear staining in crypts (compare to the adjacent normal expression in the epithelium). L: complete loss of P53 expression in epithelial cells.

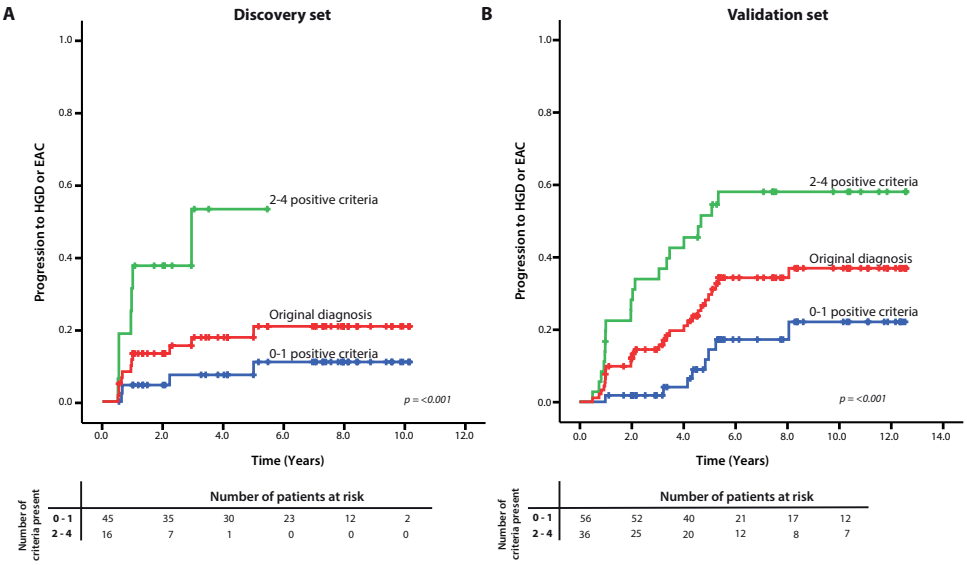

FIGURE 3: Kaplan-Meier plot, based on the first biopsy taken in the patient with low grade dysplasia (LGD), showing the cumulative estimated risk of developing high grade dysplasia or esophageal adenocarcinoma in the discovery and validation set for the original LGD diagnosis compared to the combination of the criteria (loss of surface maturation, mucin depletion, nuclear enlargement and increase in mitosis) (A, discovery set; B, validation set).

We earlier investigated prognostic value of P53 in the ProBar cohort and showed that the immunohistochemical pattern of P53 staining was related to progression (P53 expression was scored as normal expression and aberrant expression, being overexpression or loss of expression) (see Figure 2) [51]. Therefore, we here correlated P53 with the distinct histological criteria. Normal P53 staining and absence of the four histological criteria were associated with lower progression rate (5,9% in the discovery and 18.9% in the validation set) compared to aberrant P53 staining and positive histological criteria (42.9% and 68.0%, discovery and validation set respectively, see supplemental Table 7). Receiver operating characteristic (ROC) using both histological parameters and P53 were calculated, showing improved area under the curve (AUC) for combination of histological criteria and P53 (see supplemental Figure 2).

DISCUSSION

During recent years, discussion has arisen about the value of histological diagnosis of LGD as an instrument to determine surveillance interval in patients with BE. Many studies found only a weak correlation between LGD and the incidence of HGD/EAC with progression rate in patients with LGD as low as in all BE patients [39,100]. A major draw-back is that definition of LGD is inconsistent and includes a number of histological features which are difficult to interpret. Lack of a precise definition of LGD causes differences in pathological interpretation resulting in high interobserver variability [39,45,91,101,102]. Furthermore, different forms of LGD were described in the past which contributes to the complexity of the decision making for pathologists [103]. A standardized application of well-defined histological criteria would provide more objective methodology to analyze BE samples. Therefore the present study was undertaken to determine if specific histological criteria can be identified that are interpreted reliably by pathologists and whether such criteria help to improve discrimination of patients with high versus low risk for developing neoplastic progression.

First, we challenged all 12 histological criteria associated with LGD diagnosis for the interobserver agreement. As expected, even after refining the criteria by the experts, agreement between pathologist was low for most criteria. Only four of the 12 criteria, including loss of surface maturation, mucin depletion, nuclear enlargement and increase of mitosis, showed a moderate or good agreement defined by kappa values > 0.4. The complete agreement for the combination of the criteria was high in our study (75-85%, kappa value=0.46; see supplemental Table 6). The high level of agreement was confirmed in the independent set of 98 patients and was higher than in most LGD studies, with kappa values being as low as 0.11-0.27, even among expert pathologists [39,41-43]. Only few earlier studies employing selected group of highly experienced European and US pathologists could demonstrate such an improved interobserver agreement for LGD diagnosis [45,101].

Failure of maturation to the surface is suggested to be the most important characteristic of the dysplastic Barrett epithelium. Furthermore, truly dysplastic cells likely to show significant nuclear abnormalities and mitotic activity [104]. Therefore, not surprisingly, increase in mitosis, nuclear enlargement, loss of surface maturation and associated mucin depletion were predictive of progression to HGD/EAC in our patients (Table 2). When more than one criterion was present, high cumulative incidence of progression was detected (43.3% and 51.9% in the discovery and validation set respectively), while in patients with up to one criterion low progression rate was found (8.9% and 14.3% respectively). We did not further analyze other histological and cytonuclear criteria which might be useful for the diagnosis of LGD, including nuclear pleomorphism and clonal step (sharp demarcation between non-dysplastic epithelium and normal/reactive epithelium). The interobserver agreement for these criteria was weak in our hands and therefore their application for risk stratification is questionable.

Various predictive biomarkers have been studied previously in BE patients, including and especially P53. Normal expression of P53 has generally been accepted as a faint heterogeneous staining to almost no nuclear staining, while overexpression has been defined as a homogeneous strong nuclear staining in at least one crypt [51]. Loss of expression, defined as the complete absence of expression, has recently been recognized as a previously underestimated specific expression pattern associated with stop codon *TP53* mutations [105]. The use of P53 has been shown not only to reduce interobserver variation but also to improve prediction of progression [53,57,94,106]. The results of the present study indicate independent additional value of P53 to the model using the specifically defined histological features. This observation make sense by biology, since these histological criteria might result from chromosomal instability and multiplication of DNA elements leading to decreased maturation and increased mitotic activity. In BE this is frequently preceded by altered P53 function, which causes a diminished feedback-loop upon DNA damage. However, BE is a heterogeneous disease with higher rate of mutations than many common cancers and various genes are involved in development of dysplasia [107].

Clinical management of BE patients with LGD diagnosis is still under debate. International guidelines suggest either endoscopic eradication treatment or active surveillance [108-111]. The decision for one of the options might be difficult, since risks of endoscopic eradiation therapy might outweigh its benefits while surveillance might create significant burden to the patient and compliance problems [109,112,113]. Current recommendation is that the decision should be made on the individual basis, and that endoscopic therapy is appropriate in patients at highest risk of progression [88,109]. Since higher accuracy of risk prediction is improved by an expert review [45,46,88,91,114,115], confirmation by at least one expert pathologist is indicated. However it is not clear yet which of the histological features drives the LGD diagnosis in the eyes of an expert [43]. This implies significant limitations for pathologists, clinicians and patients. The problems in the interpretation come to light when observing the significant differences in progression rates reported in the literature [39,45,91,101,102]. This is also true for the geographical differences, since European pathologists might have higher interobserver-agreement compared to US pathologists [43,45,101]. In general, if all pathologists would use the same histological criteria according to standardized protocol, this could contribute to a more accurate decision-making in daily practice. Our study is intended to be the first step toward standardization of pathological assessment of BE samples. Application of a simple histological panel using the four aforementioned criteria is feasible not only for expert BE pathologists but also for pathologists with less experience in the field of BE after appropriate histological training pertaining the four specific criteria.

There are however sources of possible bias in our study population to be kept in mind. Because of the retrospective setup of the study, not all clinical data was noted in a uniform manner, although long-term follow-up data for progression was known for each patient.

Since Erasmus MC is a referral center for complex endoscopic procedures, high proportion of patients with prior HGD/EAC were found in the discovery set. Therefore, interpretation of progression rate might be limited for a more general hospital. However, this study was not intended as an incidence report but was designed to develop a new tool for improved prediction of progression in patients with LGD. Because the results derived from discovery cohort might have been impacted by the fact that majority of the progressors in this group had recurrence of LGD and a history of HGD or EAC, an independent group of patients with LGD diagnosis derived from ProBar cohort was studied [51,93,94]. ProBar patients were prospectively followed according stringent follow-up scheme and standardized endoscopy and biopsy protocol. The progression rate for the baseline LGD diagnosis in patients derived from this cohorts is comparable to recent European BE studies, being 30% [40,45,92]. Furthermore the follow-up period of some patients could be considered short, although the majority (75%) of patients without progression were followed for at least 4 years. The predictive value of the criteria however remained significant also in a more stringent analysis applying 3 year follow-up (supplemental Table 8). In summary, we have shown that specific histological criteria including loss of maturation, mucin depletion, nuclear enlargement and increase of mitosis stand out from other histological criteria showing at least moderate interobserver agreement and may be valuable to improve prediction of neoplastic progression in patients with LGD diagnosis. This finding might have great impact on the current surveillance practice, since these specific criteria could be employed by a broader pathology community. Until now, the majority of patients diagnosed with LGD according to current standards undergo intensified follow-up which is unnecessary as the diagnosis is false and hence the risk of progression low. In contrast, presence of criteria proposed in the current study indeed indicates a high risk of progression which has important management consequences such as a therapeutic intervention to ablate the dysplastic mucosal surface or intensified follow-up. In absence of these criteria, patients could be followed less rigorously. Future studies in a prospective setting are warranted to confirm our observations.

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Writing assistance:

No writing assistance or funding for writing assistance was obtained

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLE 1: Patient demographics of included patients, specified for the progressors as well as non-progressors. Progressors were defined as patients who developed high grade dysplasia or esophageal adenocarcinoma at follow-up. Non-progressors were defined as patients without neoplastic progression during follow-up.

	Discovery set			Validation set		
	Progressors (n=15)	Non-progressors (n=69)	p-value	Progressors (n=30)	Non-progressors (n=68)	p-value
Age at biopsy, Median, years (IQR)	73.5 (66.9 - 75.7)	66.6 (55.1 - 71.6)	0.001§	71.0 (66.5-74.6)	70.6 (61.5-75.8)	0.834§
Sex						
Male	12 (80.0%)	57 (82.6%)	0.726*	25 (83.3%)	51 (75.0%)	0.362°
Female	3 (20.0%)	12 (17.4%)		5 (16.7%)	17 (25.0%)	
Smoking						
Yes	1 (6.7%)	11 (15.9%)	0.543 *	5 (16.7%)	6 (9.0%)	0.268°
No	3 (20.0%)	54 (78.3%)		25 (83.3%)	61 (91.0%)	
Not available	11 (73.3%)	4 (5.8%)		0 (0%)	0 (0%)	
Use of Alcohol						
Yes	3 (20.0%)	49 (71.0%)	1.000*	24 (80.0%)	48 (70.6%)	0.331°
No	1 (6.7%)	16 (23.2%)		6 (20.0%)	20 (29.4%)	
Not available	11 (73.3%)	4 (5.8%)		0 (0%)	0 (0%)	
Esophagitis						
Yes	0 (0%)	4 (5.8%)	1.000*	29 (96.2%)	59 (86.8%)	0.169*
No	15 (100%)	65 (94.2%)		1 (3.3%)	9 (13.2%)	
Length of BE, Median (IQR)	5.0 (5.0 - 7.0)	4.0 (2.3 - 7.0)	0.039§	4.5 (3.0-7.0)	5.0 (3.0-6.0)	0.786§
Follow-up, Median, Years (IQR)	2.5 (1.5 - 4.0)	8.0 (6.3 - 9.5)	<0.001§	3.2 (1.0 – 4.7)	6.7 (4.4 – 10.4)	<0.001§
Endoscopies, Median number (IQR)	4.0 (3.0-6.0)	6.0 (4.0 - 7.0)	0.160§	4.0 (2.75 – 6.0)	6.0 (5.0 – 8.0)	<0.001§
Number of biopsies from individual patient, Median number (IQR)	2.0 (1.0-3.0)	1.0 (1.0-2.0)	0.001§	1.0 (1.0-2.0)	1.0 (1.0-2.0)	0.462§

BE, Barrett's esophagus; IQR: Inter Quartile Range; ° Pearson Chi-square test; * Fisher's exact test; § Mann-Whitney U test

SUPPLEMENTAL TABLE 2: Criteria for low grade dysplasia: refined specifications by the involved experts and interobserver agreement. Four criteria with kappa >0.4 (indicated in bolt) in the discovery set were further explored in validation set.

	Definition	Kappa	
		Discovery set*	Validation set
Loss of surface maturation	On low power, no maturation of the epithelium is seen from the proliferation zone until the surface	**0.55**	0.61
Clonal step	Abrupt transition of normal epithelium next to dysplastic epithelium	0.36	nd
Loss of polarity	More than 45 degrees of deviation of the longitudinal nuclear axis	0.29	nd
Mucin depletion	On high power, almost total to total disappearance of mucus from the surface columnar cells, dystrophic goblet cells* can be permitted	**0.51**	0.50
Stratification of nuclei	Piling of nuclei with minimum of 2 nuclei on top of each; the nuclei do not overlap	0.29	nd
Nuclear enlargement	Nuclear size at least 2x as large as nuclei of the normal not inflamed columnar epithelium	**0.41**	0.47
Form of nuclei	Elongated (pencil shaped) or round-oval nuclei	0.13	nd
Nuclear pleomorphism	Fluctuation of size and form of nuclei compared to nearby normal nuclei of the surface epithelium	0.36	nd
Hyperchromasia	Nuclei with a darker hue in comparison to the nuclei of normal columnar epithelium, nucleolus is often not recognizable anymore	0.25	nd
Prominent nucleolus	Multiple clearly enlarged nucleoli	-0.16	nd
Increase in apoptosis	More than 3 crypts in a hundred crypts with nuclear- or necrotic debris	0.13	nd
Increase in mitosis	At least one mitosis at the epithelial surface or in the neck of the crypts	**0.48**	0.46
Combination of 2 or more criteria with a kappa of >0.4	The presence of 2 or more of the criteria with at least a moderate interobserver variation in set 1 (Loss of surface maturation, mucin depletion, nuclear enlargement and increase in mitosis)	0.46	0.61

* interobserver agreement between four pathologists in discovery set was calculated using weighted kappa method (Fleiss Kappa), while in validation set kappa was calculated between 2 observers using Cohen's Kappa. **Goblet cells with the nucleus on the luminal side and the mucus on the basal side; nd: not determined

SUPPLEMENTAL TABLE 3: Percentage of agreement for the selected histological criteria for the discovery set between four pathologists and validation set between two pathologists

	Discovery set							Validation set	
	Agreement (%)							Agreement	
Observer	1vs2	1vs3	1vs4	2vs3	2v4	3vs4	Kappa	(%)	Kappa
Loss of maturation	77.7	75.8	77.8	83.0	78.7	85.3	0.55	80.61	0.612
Mucin Depletion	80.9	71.6	77.7	81.9	77.4	80.9	0.51	74.49	0.495
Nuclear enlargement	78.7	70.5	64.9	74.5	68.8	73.4	0.41	77.04	0.473
Increase of mitosis	76.6	73.7	77.7	81.9	81.7	91.5	0.48	78.06	0.460
Combination of criteria	75.5	74.7	76.6	81.9	77.4	83.0	0.46	80.61	0.613

SUPPLEMENTAL TABLE 4: Hazard ratios (HR) for individual histological criteria in an univariable Cox regression analysis for the prediction of progression to high grade dysplasia or esophageal adenocarcinoma.

	HR in univariable analysis					
	Discovery set			Validation set		
Histological criteria	HR	95% CI	P-value	HR	95% CI	P-value
Loss of surface maturation	5.51	1.79-17	<0.001	3.43	1.57-7.50	0.001
Mucin depletion	5.64	1.37-23	0.002	2.71	1.30-5.65	0.008
Nuclear enlargement	8.20	3.00-22	0.009	6.3	1.91-8.14	<0.001
Increase in mitosis	7.15	2.31-22	<0.001	2.97	1.44-6.12	0.005
Combination of criteria (ref 0-1)						
2-4 criteria present	6.72	2.15-21	<0.001	3.51	1.60-7.70	<0.001

HR: hazard ratio, CI: Confidence interval

SUPPLEMENTAL TABLE 5: Youdens index of the 4 selected criteria with an moderate interobserver variation (loss of surface maturation, mucin depletion, nuclear enlargement and increase in mitosis) for the calculation of the optimal cut-off of the number of criteria present.

Number of criteria positive	Youden index
1	0.412
2	0.411
3	0.403
4	0.33

SUPPLEMENTAL TABLE 6: Progression to High Grade Dysplasia (HGD)/ Esophageal Adenocarcinoma (EAC) for the combination of criteria assessed in the first biopsy with low grade dysplasia (loss of surface maturation, mucin depletion, increase in mitoses and nuclear enlargement.

	Discovery set			Validation set		
	0-1 criteria N = 4	2-4 criteria N = 7	Original diagnosis N=11	0-1 criteria N = 8	2-4 criteria N = 20	Original diagnosis N=28
Number of patients with progression	8.9%	43.8%	18.3%	14.3%	55.6%	30.4%
HGD/EAC incidence per patient-year	2%	22%	4%	2%	11%	5%
2-year cumulative risk of progression	4.4%	37.5%	13.1%	1.8%	27.8%	11.9%
5-year cumulative risk of progression	9.8%	43.8%	18.0%	10.7%	50.0%	26.1%

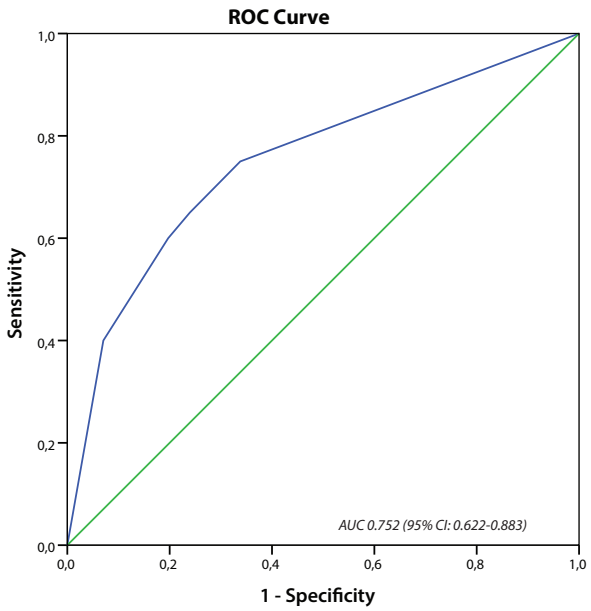
SUPPLEMENTAL TABLE 7: Correlation between the four selected histological criteria and the P53 expression. Number of patients with progression and the percentage of progression are indicated between brackets.

	Discovery set P53 expression		Validation set P53 expression	
Number of criteria	Normal	Aberrant	Normal	Aberrant
0-1 present	34 (2, 5.9%)	11 (2, 18.2%)	36 (5, 18.9%)	8 (3, 37.5%)
2-4 present	7 (2, 28.6%)	7 (3, 42.9%)	11 (3, 27.3%)	25 (17, 68.0%)

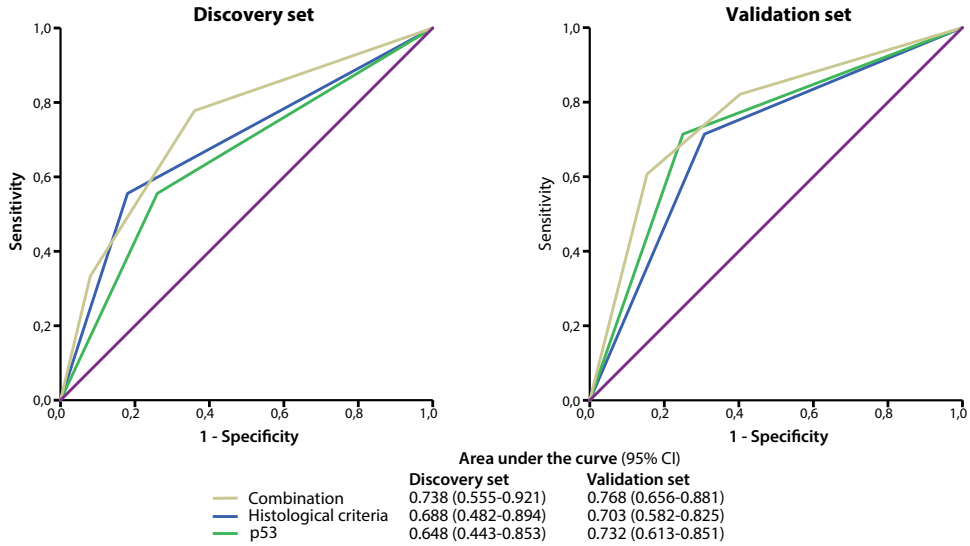
SUPPLEMENTAL TABLE 8: Hazard ratios (HR) for the combination of the histological criteria in an univariable and multivariable Cox regression analysis for the prediction of progression to high grade dysplasia or esophageal adenocarcinoma only including non-progressors with more than 3 years of follow-up after the initial low grade dysplasia diagnosis.

	HR in univariable analysis					
	Discovery set			Validation set		
Histological criteria	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Combination of criteria (ref 0-1) 2-4 criteria present	9.82	2.74-35	<0.001	4.48	1.97-10.2	<0.001
	HR in multivariable analysis					
Combination of criteria (ref 0-1) 2-4 criteria present	5.42	1.27-23	0.022	3.24	1.49-7.05	0.003

Adjusted for gender, age, length of Barrett's esophagus and esophagitis. HR: hazard ratio, CI: Confidence interval



SUPPLEMENTAL FIGURE 1: Receiver operating characteristics (ROC) curve based on the four selected criteria (loss of maturation, mucin depletion, nuclear enlargement and increase of mitosis) and the predictive value for progression from patients included in discovery set.



SUPPLEMENTAL FIGURE 2: Receiver operating characteristics (ROC) curve indicating the area under the curve (AUC) for the combination of all four criteria and P53 expression, as well as the combination of both. 95% Confidence Interval is indicated between brackets.

APPENDIX

ProBar (Progression of Barrett's esophagus) study group

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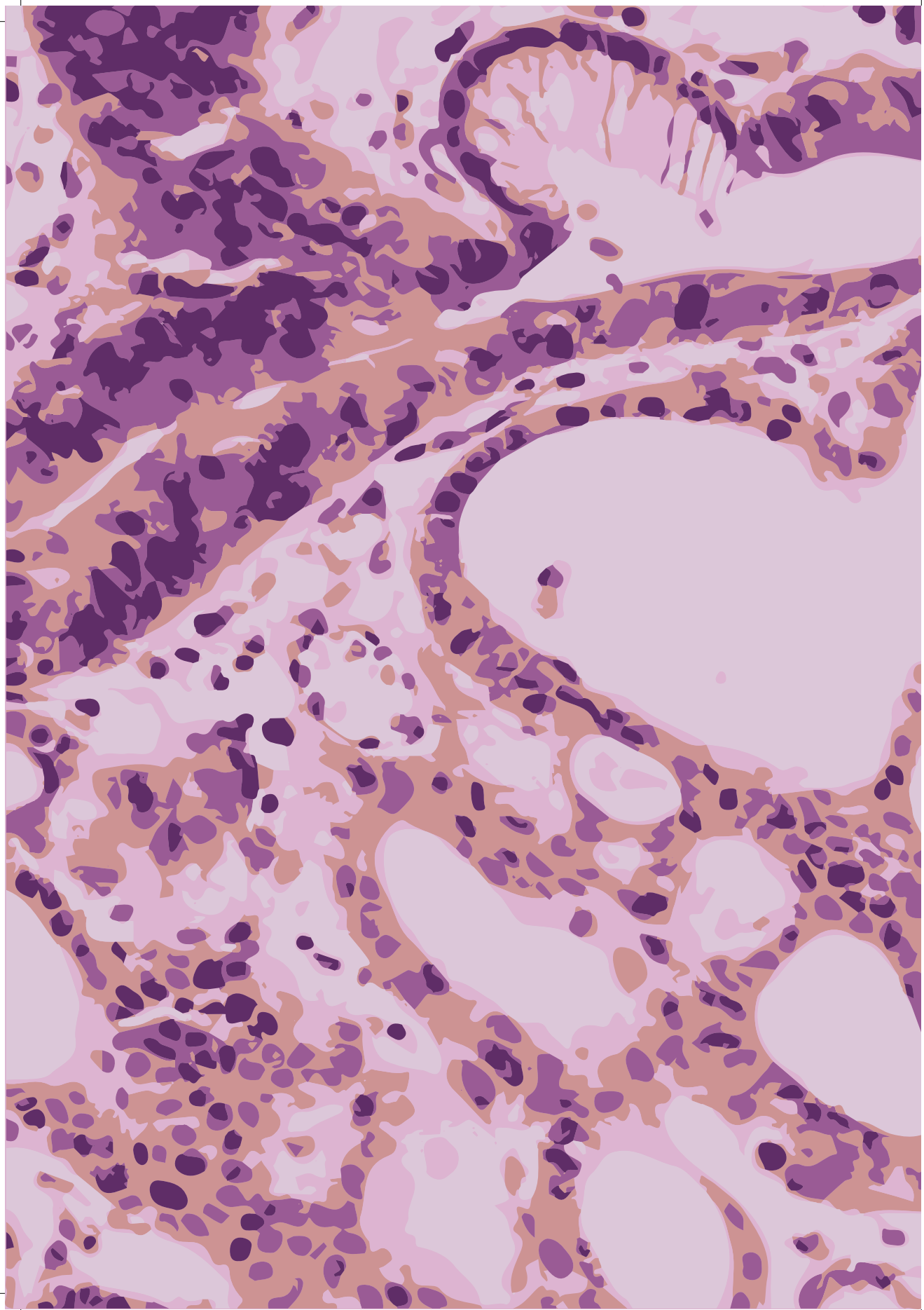

CHAPTER 4

Value of Cyclin A immunohistochemistry for cancer risk-stratification in Barrett's esophagus surveillance: A multicenter case-control study

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ABSTRACT

Introduction: The value of endoscopic Barrett's esophagus (BE) surveillance based on histological diagnosis of low-grade dysplasia (LGD) remains debated given the lack of adequate risk-stratification. The aim of this study was (I) to evaluate the predictive value of Cyclin A expression and (II) to combine these results with our previously reported immunohistochemical P53, AMACR and SOX2 data, to identify a panel of biomarkers predicting neoplastic progression in BE.

Methods: We conducted a case-control study within a prospective cohort of 720 BE patients. BE patients who progressed to high-grade dysplasia (HGD, n=37) or esophageal adenocarcinoma (EAC, n=13), defined as neoplastic progression, were classified as cases and patients without neoplastic progression were classified as controls (n=575). Cyclin A expression was determined by immunohistochemistry in all 625 patients; these results were combined with the histological diagnosis and our previous P53, AMACR and SOX2 data in loglinear regression models. Differences in discriminatory ability were quantified as changes in area under the ROC curve (AUC) for predicting neoplastic progression.

Results: Cyclin A surface positivity significantly increased throughout the metaplasia-dysplasia-carcinoma sequences and was seen in 10% (107/1050) of biopsy series without dysplasia, 33% (109/335) in LGD and 69% (34/50) in HGD/EAC. Positive Cyclin A expression was associated with an increased risk of neoplastic progression (adjusted relative risk (RR^a) 2.4; 95% CI 1.7 to 3.4). Increases in AUC were substantial for P53 (+0.05), smaller for SOX2 (+0.014), minor for Cyclin A (+0.003) and none for AMARC (0.00).

Conclusion: Cyclin A immunopositivity was associated with an increased progression risk in BE patients. However, compared to P53 and SOX2, the incremental value of Cyclin A was limited. The use of biomarkers has the potential to significantly improve risk-stratification in BE.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition of the distal esophagus in which the normal squamous epithelium is replaced by columnar epithelium containing goblet cells, as a result of chronic acid exposure [116-118]. Patients with BE have an increased risk to develop esophageal adenocarcinoma (EAC) with an estimated incidence of 0.2 to 0.5% per year [30,32,33,119]. The transition from BE to EAC is a gradual process, in which intestinal metaplasia evolves via low-grade dysplasia (LGD), to high-grade dysplasia (HGD) and finally to EAC, a cancer with an overall 5-year survival of less than 20% [78,120]. Current guidelines recommend endoscopic surveillance of BE patients to detect HGD or EAC at an early and potentially curable stage when endoscopic treatment is still feasible [35,36]. However, the applied endoscopic surveillance strategy to date based on histological diagnosis alone remains debated given the overall low incidence of neoplastic progression, and the lack of discriminative power to stratify BE patients at high risk for neoplastic progression from those at low risk.

Histological diagnosis of LGD is nowadays used for the risk assessment of neoplastic progression in BE surveillance and more intensive follow-up is recommended in LGD patients (yearly instead of every 3 years) [35,36,121]. However, diagnosis of LGD has a low predictive value, owing to sample error and a considerable inter- and intraobserver variation [41,91,122]. The use of (a panel of) biomarkers in addition to histology may improve risk stratification in BE patients, and several immunohistochemical biomarkers are under investigation. Our group previously reported on the predictive value for neoplastic progression of P53, AMACR and SOX2 in a large prospective cohort of patients with BE [51,95,123].

Another potential biomarker is Cyclin A, a protein that plays an important role in the G1-S transition of the cell cycle. Overexpression of cell-cycle related proteins, including Cyclin A, has been linked to the metaplasia-dysplasia-carcinoma sequence in BE and associated with an increased risk of neoplastic progression [48,124,125]. However, clinical validation of Cyclin A in a large prospective cohort of BE patients is still missing. In addition, there is a lack of studies testing performance of multiple biomarker simultaneously in the same cohort of BE patients.

The aim of the present study was (I) to assess the value of Cyclin A immunohistochemistry to predict neoplastic progression in a large cohort of BE patients and (II) to combine the results obtained with our previously reported P53, AMACR and SOX2 immunohistochemical data in the same prospective cohort, to identify a panel of biomarkers predictive for neoplastic progression in patients with BE.

METHODS

Study design

We conducted a case-control study nested within a large multi-center prospective cohort of 720 BE patients. All patients were included between November 2003 and December 2004 from three university medical centers and 12 regional hospitals throughout the Netherlands and received endoscopic surveillance according to the guidelines of the American College of Gastroenterology (ACG) (Appendix 1) [36]. Inclusion criterion was known or newly diagnosed BE of at least 2 cm according to the Prague C&M criteria, histologically confirmed by the presence of intestinal metaplasia on initial biopsies [126]. Patients with a history of HGD or esophageal malignancy were excluded. All endoscopic procedures were performed according to a standardized protocol, by an experienced gastroenterologist with at least several years of experience in endoscopic procedures and with interest for BE. Prior to taking biopsies, endoscopic landmarks such as the diaphragm impression, gastro-esophageal junction and squamocolumnar junction were reported. The presence of esophagitis was graded according to the Los Angeles Classification, and abnormalities were noted, including nodules, ulcers and erosions [127]. At each endoscopic procedure targeted biopsies were taken from mucosal abnormalities and quadrant biopsies were taken every 2 cm from the most distal to the most proximal part of the Barrett segment, according to the Seattle protocol [128]. Patients without dysplasia in the biopsy samples, based on histological consensus diagnosis, underwent endoscopy surveillance with biopsy sampling every three year and patients with LGD every year.

Histology

According to standard procedure, all biopsy samples were fixated with buffered formalin and embedded in paraffin. From each biopsy set, 4-micrometer thick sections were cut and stained with haematoxylin-eosin to assess the presence of BE and grade of dysplasia. After assessment of all the biopsies, the highest degree of abnormality was reported for each endoscopy. Slides were graded first by a local pathologist and secondly by an expert academic pathologist. In case of disagreement on the grade of dysplasia between the local pathologist and expert academic pathologist, the slides were reviewed by a second expert academic pathologist. Pathologists were blinded for each other's diagnosis and a final diagnosis was made if at least two pathologists agreed on the grade of dysplasia. When there was still disagreement, a panel of expert pathologists reviewed the slides and a final diagnosis was made based on consensus agreement. Given the equal surveillance strategy according to the ACG guidelines, the biopsies (n=7) with the final diagnosis of indefinite for dysplasia were included in the group of biopsies with the diagnosis of LGD.

Patient selection

We collected formalin-fixed paraffin-embedded (FFPE) material suitable for immunohistochemistry from all 720 BE patients in our cohort. However, no material or not enough material was available in 95 patients, leaving 625 patients to be included in this analysis. Patients with progression to HGD or EAC during follow-up were classified as cases and patients without neoplastic progression were classified as controls. In accordance with our previous analyses, the minimal time interval between the index endoscopy and diagnosis of HGD or EAC was nine months to prevent inclusion of prevalent cases. Immunohistochemistry was performed on the complete series of FFPE material of all surveillance endoscopies of patients who developed any form of dysplasia *i.e.* LGD, HGD or EAC during follow-up. This included the total number of biopsies taken during surveillance at different levels of the Barrett segment. In patients without any form of dysplasia during follow-up, immunohistochemistry was performed on biopsies of a random surveillance endoscopy.

Immunohistochemistry

For Cyclin A immunohistochemistry, FFPE tissue sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was done by heating in Tris buffer and endogenous peroxidase activity was blocked by incubating the slides in a solution of 0.3% hydrogen peroxide in phosphate-buffered saline. Primary antibody (Leica, Novocastra, Newcastle upon Tyns, United Kingdom: monoclonal, mouse) with a dilution of 1:200 was incubated overnight at 4 degrees Celsius. Rabbit anti-mouse (1:150; E0413, Dako, Heverlee, Belgium) was used as secondary antibody. Visualization was achieved by using the horseradish peroxidase avidin-biotin complex (HRP-ABC) method and diaminobenzidine (DAB) substrate. Finally, slides were counterstained with haematoxylin. A negative control was obtained by omission of the primary antibody. Positive nuclei in the proliferation zone of the BE epithelium were used as internal positive control. Immunohistochemical staining for P53, AMACR and SOX2 was performed as previously described [51,95,123].

Scoring of immunohistochemistry

Immunohistochemically stained slides were examined in tandem with the haematoxylin-eosin stained slides to determine Cyclin A, and previously P53, AMACR and SOX2 expression in areas with dysplasia [51,95,123]. Nuclear Cyclin A expression was scored on a two-point scale; negative or positive expression. The surface cells were counted up to a maximum of 600 cells to determine the percentage of Cyclin A positive cells. Only surface cells with strong nuclear staining were considered as positive. The epithelial surface was defined as the columnar cells at the luminal side of the biopsy, as described previously [129]. Based on published data, a cut-off value of 1% or more was used for Cyclin A positivity [125]. Cyclin A expression was

scored in BE epithelium with the highest percentage of positive Cyclin A cells and in biopsy series with dysplasia, Cyclin A expression was scored in the dysplastic area. After scoring all biopsies, the highest degree of abnormality was reported for each surveillance endoscopy. All stained slides were scored by two independent expert investigators who were blinded for long-term outcome as well as each other's results. When there was disagreement between the two investigators, slides were reviewed by an experienced academic pathologist (KB or MD) and final diagnosis was made if two investigators agreed on the extend of Cyclin A expression.

P53, AMACR and SOX2 expression was scored as previously described [51,95,123]. Briefly, nuclear P53 and cytoplasmatic AMACR expression were scored on a three-point scale (P53; normal expression, overexpression or loss of expression and for AMACR; no expression, mild expression or strong expression). Only intense nuclear staining for P53 was scored as overexpression and aberrant P53 expression was defined as either overexpression or complete loss of expression in at least one gland. Nuclear SOX2 expression was scored on a two-point scale; positive or loss of expression. Positive expression included strong as well as weak nuclear SOX2 positivity and was interpreted as normal expression. Loss of SOX2 expression in a cluster of glands, excluding BE glands containing many goblet cells was defined as aberrant SOX2 expression.

Ethics

The study protocol was approved by the institutional review board of the Erasmus University Medical Center, including those of all participating hospitals. Before the first endoscopy, written informed consent was obtained from all 720 BE patients.

Statistical analysis

Patient characteristics of cases and controls were compared using Mann-Whitney *U*-tests for continuous variables and χ^2 tests for categorical variables. To compare Cyclin A expression in biopsy series of cases and controls with different grade of dysplasia, the Mann-Whitney *U*-tests test and Kruskal-Wallis test were used, thereby ignoring that multiple biopsy series could be from the same patient. Neoplastic progression was defined as the development of HGD or EAC at least 9 months after inclusion in the study, and follow-up time was defined as the time between two consecutive surveillance endoscopies. The value of Cyclin A immunohistochemistry to predict neoplastic progression was estimated in loglinear regression models. Previous stained slides for P53, AMACR and SOX2 expression in the same cohort of BE patients were re-evaluated in this study to explore the classification performance of different combinations of biomarkers for predicting neoplastic progression in BE. Because immunohistochemical staining was not performed on all biopsy series, data were split up by endoscopy (1,243 in 575 controls, 142 in 50 cases). Loglinear models were

used to calculate relative risks (RRs) and 95% Confidence Intervals (CIs) with the logarithm of follow-up time (time between two consecutive endoscopies) as offset variable. In multivariable analysis we adjusted for gender, age, BE length and esophagitis to estimate adjusted RRs and 95% CIs. For each of the biomarkers the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) was calculated. The areas under the Receiver Operating Characteristic (ROC) curves for neoplastic progression were calculated for the individual markers as well as for the comparison between a selection of models, in which the studied biomarkers were included or excluded. These included pathological diagnosis of grade of dysplasia alone, pathological diagnosis in combination with P53 and SOX2 immunohistochemistry and pathological diagnosis in combination with P53, SOX2 and Cyclin A immunohistochemistry. The incremental value of each biomarker was calculated by the change in AUC after exclusion of the concerning biomarker in the 'fully adjusted model' (model including histological diagnosis, Cyclin A, P53, AMACR and SOX2 immunohistochemistry) as described earlier [130]. Interobserver agreement for Cyclin A expression was determined by Cohen kappa statistics. Kappa value of below 0.21 were considered 'poor', 0.21 to 0.40 'fair', 0.41 to 0.60 'moderate', 0.61 to 0.8 'substantial', and above 0.81 'very good'[131]. Two sided *p* values of <0.05 were considered statistically significant. Data were analyzed using SPSS statistical software (V.21.0; IBM SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

Six hundred and twenty-five patients with BE were included in this study (74% men, median age of 60 years (interquartile range (IQR) 53-69)) and followed for a median duration of 6.7 years (IQR 5.0-7.4). Thirty-seven (6%) patients developed HGD and 13 (2%) patients developed EAC during surveillance after a median follow-up of 3.2 years (IQR 1.9-5.3). These 50 (8%) BE patients with neoplastic progression were classified as cases and the remaining 575 (92%) patients without neoplastic progression were classified as controls. Cyclin A expression was scored separately and subsequently correlated with histological diagnosis and expression of P53, AMACR and SOX2 in biopsy series of 1,432 endoscopies: 189 endoscopies were performed in 50 cases and 1,243 endoscopies in 575 controls. Biopsy series were defined as the total number of biopsies from one endoscopy and the highest degree of abnormality was reported for each surveillance endoscopy after evaluation of all biopsies taken at that respective endoscopy procedure. Except for a smaller number of endoscopies, a higher number of biopsies per endoscopy, longer BE length and more frequent diagnosis of LGD at baseline there were no significant differences between the cases and controls (Table 1).

Histology

Consensus histology assessments included, 1,050 (73%) biopsy series with non-dysplastic BE (NDBE), 335 (23%) with LGD, 34 (3%) with HGD and 13 (1%) with EAC. The local pathologist and expert academic pathologist disagreed on grade of dysplasia in 421 (29%) biopsy series and these samples were reviewed by a second expert pathologist (kappa-value of 0.34; 95% CI 0.32 to 0.36). In 22 (19%) biopsy series there was still disagreement and a second expert pathologist or a panel of expert pathologists reviewed the slides for a final diagnosis. The presence of LGD was more frequent in biopsy series of cases (47%) than in biopsy series of controls (22%) and was associated with an increased risk of neoplastic progression after adjusting for gender, age, BE length and esophagitis (adjusted RR of 3.9; 95% CI 2.8 to 5.4), with an AUC of 0.62 (95% CI 0.58 to 0.68) (Table 2 and Figure 1). The sensitivity of histological diagnosis of LGD for predicting neoplastic progression was 47%, with a specificity of 78%. The PPV and NPV were respectively 20% and 93% (Table 3).

TABLE 1. Baseline characteristics of cases and controls

	Controls n = 575	Cases n = 50	*p* Value
Follow-up, Median, years (IQR)	6.5 (5.2-7.2)	3.2 (1.9-5.3)	<0.001
Endoscopies, Median number (IQR)	4 (4-5)	3 (2-4)	<0.001
Biopsies available, Median number per endoscopy (IQR)	6 (4-9)	9 (6-12)	<0.001
Age, Median, years (IQR)	60 (53-69)	65 (56-71)	0.103
Male sex	419 (73%)	41 (82%)	0.160
Alcohol use			
Never	66 (12%)	6 (12%)	0.981
Former	52 (9%)	5 (10%)	
Current	445 (79%)	39 (78%)	
Smoking			
Never	189 (34%)	12 (24%)	0.362
Former	256 (45%)	25 (50%)	
Current	118 (21%)	13 (26%)	
Reflux symptoms	172 (30%)	19 (38%)	0.265
Barrett diagnosis			
≤ 1999	231 (41%)	16 (32%)	0.473
2000-2002	197 (34%)	19 (38%)	
2003-2004	141 (25%)	15 (30%)	
Barrett length, Median, cm (IQR)	4 (3-6)	5 (4-7)	0.010
Low-grade dysplasia at baseline	88 (15%)	24 (48%)	<0.001
Esophagitis	109 (19%)	14 (30%)	0.104

IQR, Interquartile range.
Patients with neoplastic progression were classified as cases and patients without neoplastic progression were classified as controls. Mann-Whitney *U*-test and chi-squares test were used to compare the characteristics of cases and controls.

Cyclin A immunohistochemistry

A positive Cyclin A expression was seen in 250/1,432 (17%) of the biopsy series. The interobserver agreement for Cyclin A expression was moderate with a kappa-value of 0.46 (95% CI 0.43 to 0.49). The observers disagreed on Cyclin A surface expression in 278 (19%) biopsy series (Table 4). Cyclin A surface positivity was seen in 107 (10%) biopsy series without dysplasia, and was more common in dysplastic BE, including 109 (33%) biopsy series with LGD, 26 (76%) biopsy series with HGD and eight (62%) with EAC (p<0.001). Positive Cyclin A surface expression was more common in biopsy series of cases (32%) than in biopsy series of controls (14%), and it was associated with an increased risk of neoplastic progression with a RR of 2.7 (95% CI 1.9 to 3.8). This association remained after adjusting for gender, age,

BE length and esophagitis (adjusted RR 2.4; 95% CI 1.7 to 3.4) and was particularly seen in biopsy series with LGD (adjusted RR of 5.8; 95% CI 3.7 to 9.0) (Table 2). In per-biopsy analysis, Cyclin A had an AUC of 0.59 (95% CI 0.54 to 0.64) for predicting neoplastic progression with a sensitivity of 32%, a specificity of 86%, a PPV of 21% and a NPV of 92% (Table 3).

TABLE 2: Histology and Cyclin A immunohistochemistry in biopsy series of cases and controls

Variable	Controls n = 1,243	Cases n = 142	RR (95% CI)	RR[a] (95% CI)
Histology				
ND	975 (78%)	75 (53%)	Reference	Reference
LGD	268 (22%)	67 (47%)	4.2 (3.0 to 5.8)	3.9 (2.8 to 5.4)
Cyclin A expression				
< 1%	1073 (86%)	96 (68%)	Reference	Reference
≥ 1%	170 (14%)	46 (32%)	2.7 (1.9 to 3.8)	2.4 (1.7 to 3.4)
Histology and Cyclin A expression				
ND and < 1% Cyclin A positivity	883 (71%)	60 (42%)	Reference	Reference
LGD and < 1% Cyclin A positivity	190 (15%)	36 (25%)	3.8 (2.5 to 5.8)	3.5 (2.3 to 5.3)
ND and ≥ 1% Cyclin A positivity	92 (8%)	15 (11%)	2.0 (1.2 to 3.6)	1.7 (0.9 to 3.0)
LGD and ≥ 1% Cyclin A positivity	78 (6%)	31 (22%)	6.4 (4.1 to 9.9)	5.8 (3.7 to 9.0)

The highest degree of abnormality was reported for each endoscopy after examining all biopsies.
RR, relative risk as calculated from a log-linear regression model; CI, confidence interval; ND, no dysplasia; LGD, low-grade dysplasia.
[a] RR adjusted for gender, age, BE length and esophagitis.

TABLE 3: Performance of each individual marker for predicting neoplastic progression

Biomarker	Sensitivity	Specificity	PPV	NPV	AUC (95% CI)
Low-grade dysplasia	47%	78%	20%	93%	0.62 (0.58 to 0.68)
Cyclin A positivity	32%	86%	21%	92%	0.59 (0.54 to 0.64)
Aberrant P53	51%	87%	30%	94%	0.69 (0.64 to 0.74)
Strong AMACR	11%	96%	25%	90%	0.53 (0.48 to 0.59)
Loss of SOX2	25%	93%	29%	92%	0.60 (0.55 to 0.65)

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the ROC curve; CI, confidence interval
The highest degree of abnormality was reported for each endoscopy after examining all biopsies.

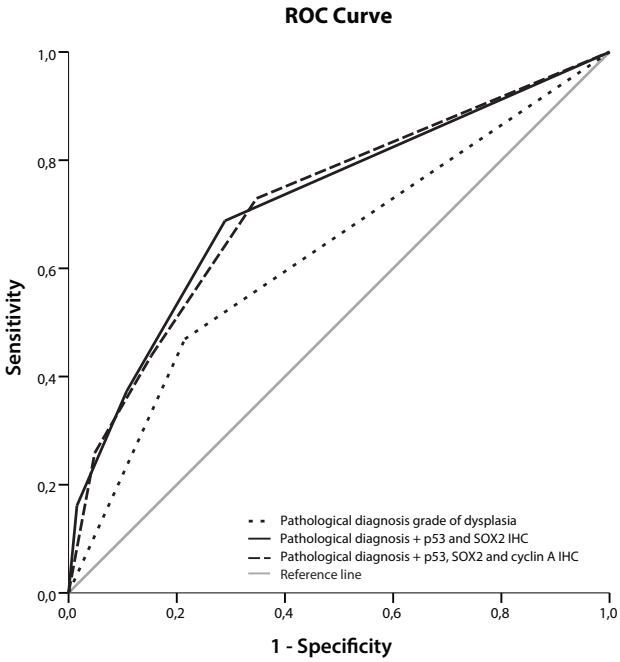


FIGURE 1: Receiver operating characteristic (ROC) comparing different biomarker models with the basic pathological diagnosis of grade of dysplasia. Area under the curve (AUC) for predicting neoplastic progression was calculated (pathological diagnosis grade of dysplasia AUC of 0.62 (95% CI 0.58 to 0.68), pathological diagnosis + P53 and SOX2 immunohistochemistry AUC of 0.72 (95% CI 0.67 to 0.77) and pathological diagnosis + P53, SOX2 and Cyclin A immunohistochemistry AUC of 0.72 (95% CI 0.67 to 0.77)).

P53, AMACR and SOX2 immunohistochemistry and incremental value of Cyclin A

The pattern of P53, AMACR and SOX2 expression were previously studied and discussed elsewhere [51,95,123]. Aberrant P53 expression, as well as strong AMACR expression and aberrant SOX2 expression were more common in biopsy series of cases than in biopsy series of controls (P53; 51% vs. 13%, AMACR; 11% vs. 4%, SOX2; 25% vs. 7%) and were associated with an increased risk of neoplastic progression with adjusted RR of 5.6 (95% CI; 4.0 to 7.8) for aberrant P53 expression, 2.8 (95% CI; 1.6 to 4.8) for strong AMACR expression and 4.4 (95% CI; 3.0 to 6.5) for aberrant SOX2 expression, respectively (supplementary Table 1) (Table 3). The highest risk of neoplastic progression was detected in patients with LGD and

concurrent aberrant P53 expression (adjusted RR of 9.9; 95% CI 6.6 to 14.9) (supplementary Table 1). The addition of P53 immunohistochemistry improved the AUC compared to the histological diagnosis alone (from AUC 0.62 to AUC 0.70; 95% CI 0.66 to 0.76).

Next, we combined the information on histology, Cyclin A, P53, AMACR and SOX2 immunohistochemistry in a fully adjusted model for predicting neoplastic progression in BE (Table 5). Aberrant P53 expression showed the highest change in AUC (0.05), to a lesser extent aberrant SOX2 expression (0.014) and histological diagnosis of LGD (0.005). The biomarkers Cyclin A and AMACR only showed a minimal drop or no drop in AUC after exclusion (Cyclin A: 0.003 and AMACR: 0.0). Importantly, the addition of SOX2 slightly improved the AUC compared with the model including only histological diagnosis and P53 immunohistochemistry (from AUC 0.70 to AUC 0.72; 95% CI 0.67 to 0.77) (Figure 1).

TABLE 4: Interobserver agreement for Cyclin A expression

Cyclin A surface positivity	< 1%	≥ 1%	ƙ value
< 1%	958 (67%)	122 (8%)	0.46
≥ 1%	156 (11%)	196 (14%)	

The highest degree of abnormality was reported for each endoscopy after examination of all biopsies.
Cohen ƙ statistics were used to determine interobserver agreement.

TABLE 5: Fully adjusted model with histology, Cyclin A, P53, AMACR and SOX2 immunohistochemistry in biopsy series of cases and controls.

Variable	RR[a] (95% CI)	Change in AUC[b]
Low-grade dysplasia	1.8 (1.2 to 2.6)	0.005
Cyclin A positivity	1.4 (1.0 to 2.1)	0.003
Aberrant P53	3.7 (2.6 to 5.4)	0.050
Strong AMACR	1.3 (0.8 to 2.3)	0.000
Loss of SOX2	2.2 (1.4 to 3.4)	0.014

[a] RR adjusted for gender, age, BE length and esophagitis and all the other biomarkers
[b] Calculated drop of AUC after exclusion of the concerning biomarker compared to AUC of the total model (AUC of 0.734; 95% CI 0.687 to 0.780)

DISCUSSION

In this large case-control study we evaluated the value of Cyclin A expression for predicting neoplastic progression in patients with BE. These results were combined with our previously reported P53, AMACR and SOX2 immunohistochemical data within the same cohort using AUC in ROC analysis, to explore the classification performance of different combinations of biomarkers. This modeling is a valuable tool for the overall judgment of the incremental value of the biomarkers studied but not intended as an exact analytic method [130]. Cyclin A surface positivity significantly increased throughout the metaplasia-dysplasia-carcinoma progression steps and was associated with an increased risk of neoplastic progression. However, the incremental value of Cyclin A expression was limited compared to histological diagnosis of LGD, P53 and SOX2.

Surveillance of BE patients is under significant debate given the lack of discriminative tools for adequate risk stratification. Additionally, with the introduction of minimally invasive endoscopic therapy and the evidence of cancer prevention by radiofrequency ablation in patients with LGD, there is an increasing need for accurate dysplasia detection during BE surveillance [108,110]. Previous studies demonstrated repeatedly the value of LGD as a risk factor for neoplastic progression, albeit with a low predictive value due to sampling error and considerable interobserver variation [30,32,41,91,121,122]. Even though the predictive value of LGD increases with consensus of multiple pathologists, approximately one-third of the patients with BE are diagnosed with LGD during surveillance, whereas the 5-year cumulative incidence of neoplastic progression is only between 5%-30% in this group [42,45,122]. Although the result of our study support the use of LGD diagnosed by expert GE pathologists, as indicator for increased risk of neoplastic progression, its sensitivity is only 47% and specificity 78%, despite using a consensus diagnosis of dysplasia. These results exemplify the interest in identifying molecular biomarkers to improve risk stratification and eventually cost-effectiveness of BE surveillance.

In the present study, Cyclin A expression was confined to the base of the crypts in normal columnar gastrointestinal epithelium, as well as in most non-dysplastic BE. With increasing grades of dysplasia the expression of Cyclin A progressively shifted towards the surface epithelium. The percentage of biopsy series with a positive Cyclin A surface expression increased from 10% in non-dysplastic BE to 62% in biopsy series with EAC, which corresponds to previous studies [124,125]. A recent study identified Cyclin A expression as one of a three-biomarker panel which provides a more accurate and objective diagnosis of dysplasia in BE [124]. Our results confirmed the correlation between dysplasia and Cyclin A expression and hence potential as diagnostic tool for dysplasia detection.

Positive Cyclin A surface expression was detected more frequently in cases than in controls, and was significantly associated with an increased risk of developing HGD or EAC (adjusted RR 2.4; 95% CI 1.7 to 3.4), particularly in dysplastic BE. The results of previous

studies evaluating the value of Cyclin A expression for predicting neoplastic progression are conflicting. A small case-control study showed that Cyclin A surface expression was significantly associated with an increased risk of neoplastic progression (OR 7.6; 95% CI 1.6 to 37.0), whereas a more recent larger population-based study could not confirm this correlation and only found a trend towards an increased risk of progression, which eventually lost significance in a multivariate analysis (OR 1.32; 95% CI 0.66 to 2.66)[48,125]. These conflicting results might be explained by a rather challenging interpretation of Cyclin A immunohistochemistry. We found a moderate interobserver agreement with a kappa value of 0.46. This is low compared to the interobserver agreement of the other biomarkers P53 and SOX2 (kappa values between 0.70 and 0.86) [51,55,95].

The biomarker with the greatest body of evidence remains aberrant P53 expression (adjusted RR in fully adjusted model of 3.7 (95% CI 2.6 to 5.4), change in AUC 0.05) and to a lesser extent aberrant SOX2 expression (change in AUC 0.014). Cyclin A positivity showed only a minimal drop in AUC after exclusion (0.003). These findings might have important and clinically relevant implications. Assessment of P53 and SOX2 are promising to select high-risk patients for either intensified surveillance or ablation therapy and may eventually contribute to a more cost-effective management. Although routine P53 and SOX2 staining and assessment incur higher costs than histology alone, application of this panel of biomarkers has the potential to reduce the overall costs related of Barrett surveillance. Patients at low-risk of neoplastic progression, *i.e.* the majority of the patients with LGD, might be followed-up less intensively with the potential to eventually discharge them. However, a more detailed cost-effectiveness analysis should be performed to evaluate the economic value of P53 and SOX2 immunohistochemistry, which is beyond the scope of this study.

Our study has several strengths. The large cohort of BE patients was prospectively followed-up according to a stringent scheme during a long follow-up time, clinical, endoscopic and pathological data were collected. Additionally, a standardized endoscopy and biopsy protocol was used. All stained slides were assessed by at least two experienced observers blinded for clinical outcome and in case of disagreement an expert pathologist reviewed the slides for final diagnosis. Another major strength of this study was that we tested multiple biomarkers in the same cohort of BE patients so we could identify the smallest panel of biomarkers with the highest predictive value for neoplastic progression, and which can be performed on routine clinical collected FFPE tissue.

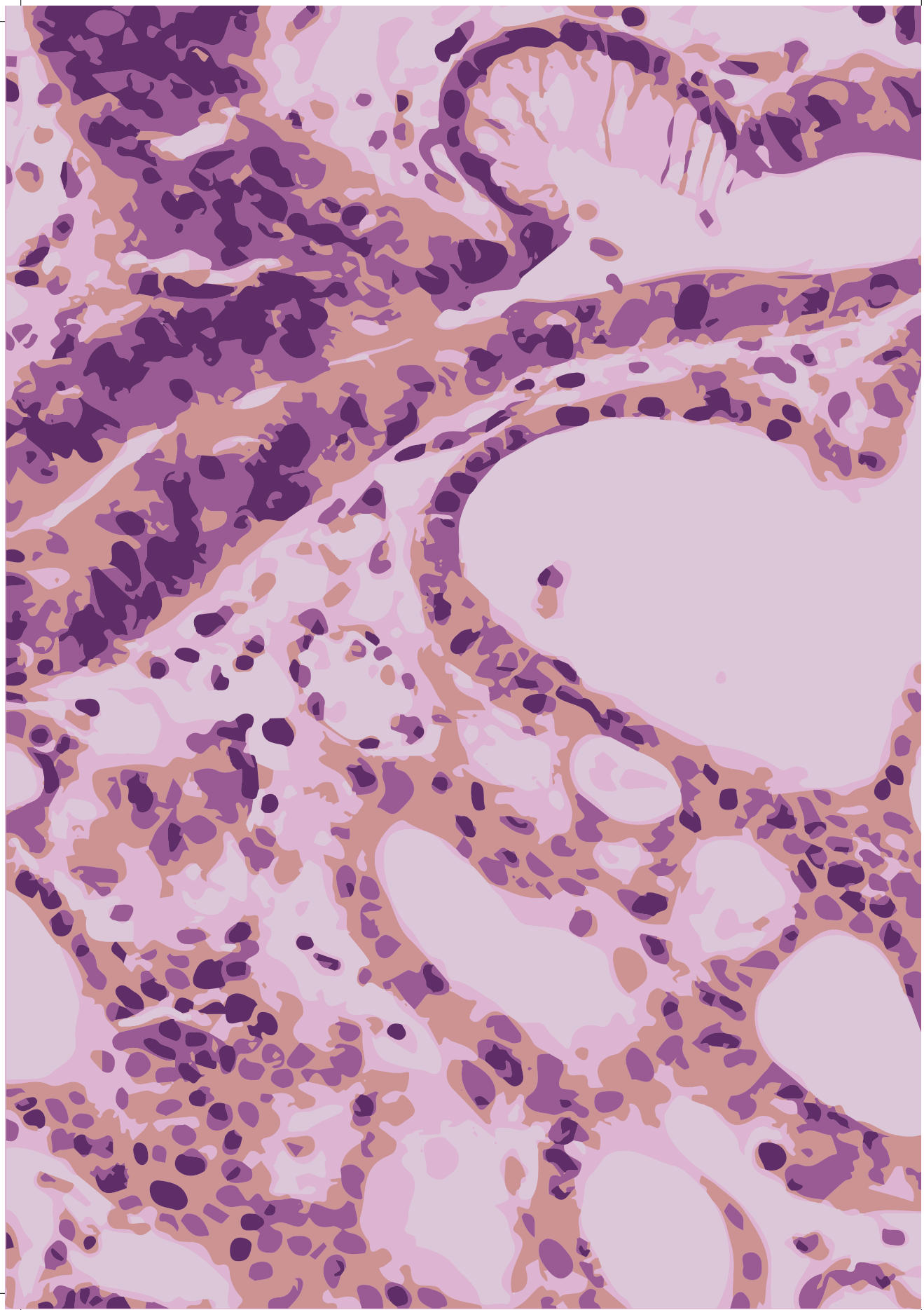
Our study also has some limitations. Although immunohistochemistry is an established clinical examination method and easily applicable to standard clinical pathological laboratories, the scoring of the expression is a subjective assessment. It will require standardization of processing and scoring for reliable routine clinical application. In spite of this, our previous studies have shown good interobserver agreement for both P53 and SOX2 and they were relatively simple and straightforward to interpret [51,95]. Further validation

of this panel of biomarkers in large prospective studies is required to confirm our findings. Secondly, as all patients with BE, the patients considered as controls in this study still have the potential to progress to HGD or EAC during the future follow-up. However, since their median follow-up time was 6.5 years (which is more the twice the follow-up time of the cases), and the incidence of progression in only 2,6/1000 patients per year, the chance of progression in the controls is slim [32].

In conclusion, Cyclin A surface expression was associated with an increased risk of neoplastic progression in BE patients, but its ability to predict neoplastic progression is limited compared to the biomarkers P53 and SOX2. The use of biomarkers has the potential to significantly improve risk-stratification in Barrett surveillance and hence the cost-effectiveness of Barrett surveillance programs.

PART III

Esophageal adenocarcinoma



CHAPTER 5

Tumor budding is prognostic for lymph node metastasis and survival in patients with pT1b esophageal adenocarcinoma

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Submitted

ABSTRACT

Introduction: Clinical management of patients with esophageal adenocarcinoma with submucosal invasion (pT1b EAC) is based on estimated risk for developing lymph node metastasis (LNM), which is inaccurate using current standard histological tumor characteristics. Tumor budding (TB) has shown to be prognostic of LNM in colorectal cancer, but its value for early EAC has not been established yet.

Methods: In the present study we compared different manual TB scoring methods (described by Ueno, Ohike and Thies), as well as automated digital image evaluation, with the goal to select and validate the most reproducible and prognostic TB scoring system for patients with pT1b EAC.

Results: Firstly we investigated 25 cases, demonstrating a good to excellent interobserver agreement for TB scoring using methods according to Ueno and Ohike. In the validation cohort of 103 pT1b EAC, TB according to Ohike method was prognostic for LNM and survival, also in multivariable Cox regression analysis employing all known histological risk factors (Odds Ratio LNM 3.51 (95% CI 1.05-11.68, p-value 0.041); Hazard Ratio Overall Survival 2.20 (95CI 1.17-4.12, p-value: 0.014); Hazard Ratio Disease Free Survival 2.99 (95% CI 1.22-7.35, p-value 0.017)). Additional immunohistochemistry (pankeratin & desmin double staining) did not improve interobserver agreement and was not independently predictive for LNM status.

Conclusion: Our study shows that TB scoring according to Ohike is highly reproducible, and independently predictive of LNM and survival in pT1b EAC. TB is recommended to be implemented in the pathological assessment to improve prediction of LNM and adjustment of the therapeutic decision making in patients with pT1b EAC.

INTRODUCTION

In contrast to patients with advanced esophageal adenocarcinoma (EAC) who have a 5-year survival of 30-50% after curative treatment, prognosis of patients with early invasive EAC (pT1) is favorable with a 5-year survival of 80% [132]. The outcome in these patients is mainly determined by the presence of lymph node metastasis (LNM) [133]. In patients with mucosal invasion (pT1a) only the risk of LNM is very low and peri-operative risks outweigh the risk of metastasis [134-136]. Therefore it is recommended that patients with well differentiated pT1a EAC with diameter <2cm are treated by endoscopic resection only [37,66,137].

Risk of LNM is considerably higher in EAC with submucosal invasion (pT1b), presumably because of the presence of small lymph- and blood vessels in the submucosa connected with the regional lymph nodes [138-143]. Management of patients with EAC staged as pT1b tumor in an endoscopic resection specimen is determined by tumor characteristics such as size, depth of invasion, differentiation grade, lympho-vascular invasion and status of the resection margins. In patients with unfavorable tumor characteristics, the prevalence of LNM increases from 3-10% to 22-45% [66-69]. However, individual risk of LNM is difficult to predict and additional biomarkers are therefore needed to improve patient stratification. Identifying prognostic markers in early invasive cancer stage such as pT1b EAC is challenging since sufficient power is difficult to achieve due to low incidence of LNM and cancer related deaths in these patients.

One of the most promising biomarkers for LNM in e.g. colorectal cancer is tumor budding (TB) [144,145]. TB is usually assessed at the invasive tumor front and defined as a single tumor cell or a cluster of at most four tumor cells without signs of glandular differentiation. Little is known so far about the impact of TB in pT1b EAC. Recent studies on histological risk factors for the development of LNM did not include TB [68,69,138,142,143,146] and only one previous study investigated TB in a cohort of patients with either pT1a or pT1b EAC [147]. In contrast, comprehensive knowledge is available on TB in advanced colorectal (CRC) and gastric cancer [148-159]. Furthermore, multiple studies have shown predictive value of TB for presence of LNM in pT1 CRC, and TB is included in the CRC management guidelines in Japan and USA [160,161]. Its clinical utility in gastrointestinal cancers is however limited due to different methods for assessing TB [133,147,162-169]. A recent consensus meeting concluded that standardized, evidence-based TB method is needed for future reporting in the clinical practice and is crucial for future reproducible interpretation of TB in clinical trials [170].

Given substantial variations of LNM risk in pT1b EAC and insufficient prognostic power using standard tumor characteristics, we aimed to determine if TB could be of value in this setting. Different standard methods of TB assessment were compared in the discovery cohort and most informative and reliable TB methods were validated in an independent set of pT1b EAC. In addition, digital tumor bud count (DTBC) for the assessment of TB was compared to the standard manual pathological evaluation.

METHODS

Patient selection and patient material

All consecutive patients with a pT1b EAC on the original pathological report, treated from 1989-2014 at Erasmus MC - University Medical Center Rotterdam and three community hospitals (IJsselland Hospital (Rotterdam), Isala Hospital (Zwolle) and Catharina Hospital (Eindhoven)) were retrospectively identified using the Registry of the Netherlands Comprehensive Cancer Organization (IKNL). Formalin fixated paraffin embedded (FFPE) material and the original H&E slides of the endoscopic resection or radical esophagectomy specimens were collected. Clinical and pathological data were retrospectively reviewed, including age at treatment, time of disease recurrence, date of death, tumor location and diameter. Tumors of which the representative slides could not be retrieved from the archives or in which submucosal invasion could not be confirmed were excluded. Also, only patients who were treated by surgical resection or endoscopic resection were included in the study, while patients treated with adjuvant or neoadjuvant chemotherapy or radiotherapy were excluded. To ensure reliable pathological classification all cases were reviewed by two experienced GI-pathologists (FK, KB). Histological assessment included depth of submucosal invasion, (lympho-) vascular invasion, tumor grading according to the WHO classification [171] and pathological tumor staging according to the TNM-classification as described by the UICC (Union Internationale Contre le Cancer, 2010) [75].

Study design and methods for tumor budding count

Different classification systems for TB including Ueno, Ohike and Thies method were described in detail in earlier publications related to gastrointestinal and esophageal carcinomas [147,165,168]. These TB methods were compared in the discovery cohort and the results validated in an independent group of patients with pT1b EAC (see Figure 1, study design). For the discovery cohort, 30 consecutive patients from the Erasmus MC were used (20 patients without LNM and 10 patients with LNM) based on the primarily available material. All other patients were included in the validation cohort. Two experienced GI-pathologist (KB and FK) independently assessed TB, on H&E and pankeratin stained slides.
Briefly, a tumor bud (TuB) was defined as presence of a single tumor cell or a cluster of up to four tumor cells, completely surrounded by stroma and lacking glandular formation. For the Ueno method the invasive front was scanned with a 10x objective lens to determine the field (0.785 mm^2 =20x objective) with the highest number of tumor buds. Total number buds were counted in this single hotspot area [165]. For the Ohike method the entire invasive front was screened and number of fields (0.785 mm^2 =20x objective) with at least 5 buds were counted [172]. For the Thies method counting of buds was done in one area (0.189 mm^2

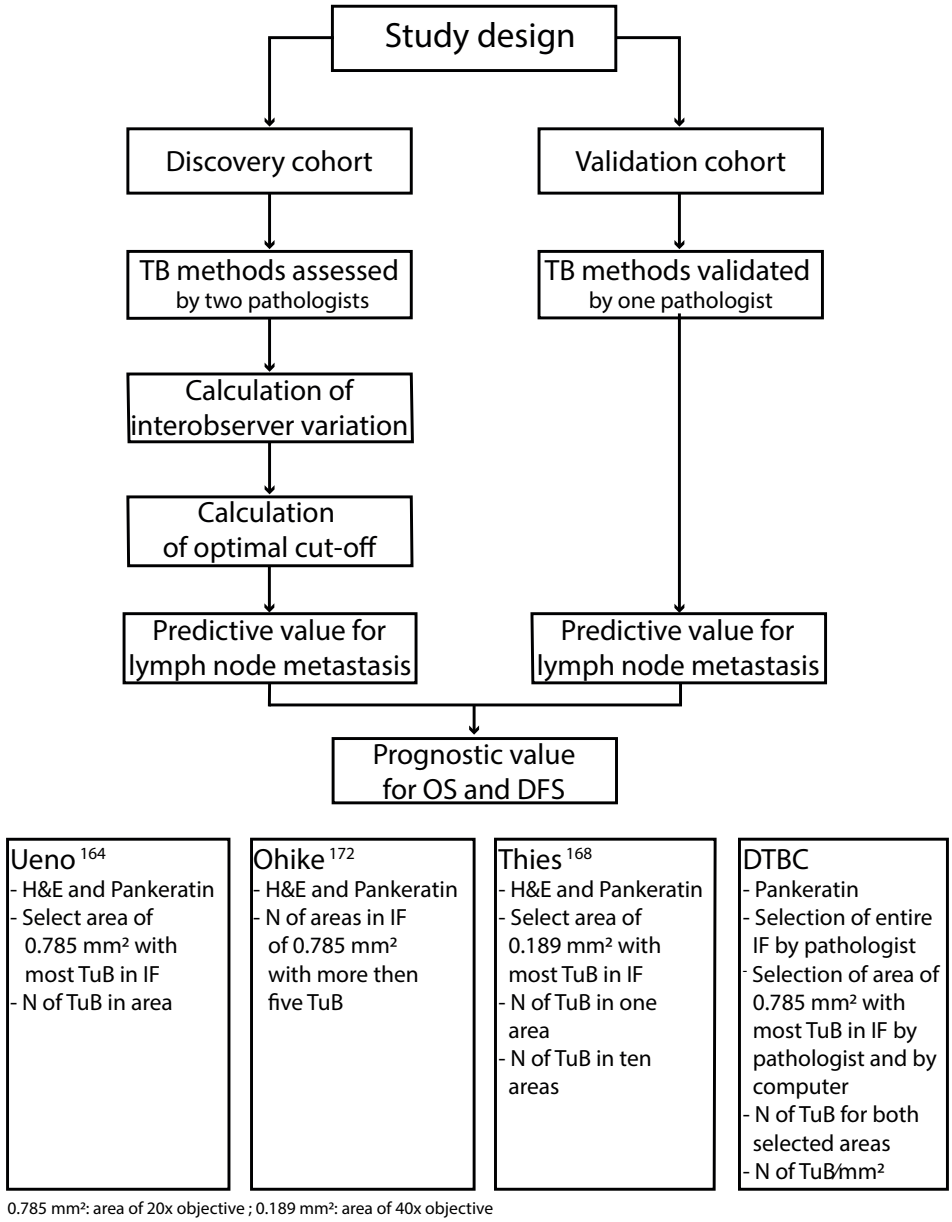


FIGURE 1: Flow diagram depicting study design. Different methods for assessment of Tumor Budding (TB) were compared, including standard manual assessment according to Ueno [164], Ohike [172] and Thies [168], as well as using digital tumor budding count (DTBC). Best manual TB methods and DTBC were validated in an independent cohort. N: Number; OS: Overall Survival; DSF: Disease Free Survival; H&E: Hematoxylin and Eosin stained slides; TuB: Tumor Buds; IF: Invasive Front

= 40x objective) as wells as in 10 of these hotspots at the invasive front [168]. Cut-off values for high vs low TB were defined according to the earlier publications: Ueno (H&E) method: five or more buds [165]; Ohike (H&E) method: three or more budding fields [172]; Thies ten fields (pankeratin) method: 130 or more buds [168]. Optimal cut-offs for all other methods (Ueno and Ohike (pankeratin based), Thies one field (H&E andpankeratin based), Thies ten fields (H&E based) were calculated in the discovery cohort.
Next, interobserver agreement was determined for all TB methods. For those methods with the highest agreement, predictive value for LNM was calculated separately on the discovery and validation cohort. The prognostic value for overall survival (OS) and disease free survival (DFS) was calculated on the entire patient group.

Digital assessment of tumor budding

Besides the standard visual assessment by the pathologist using microscope, TB was analyzed by digital tumor bud count (DTBC) (Visiomorph, Visiopharm, Hoersholm, Denmark). All pankeratin stained slides were digitalized (Nanozoomer 2.0HT, Hamamatsu, Almere, the Netherlands) with a 40x lens in a single layer and imported in Visiomorph. One of the participating pathologists (FK) checked the images manually to ensure good quality images and delineated the invasive border as well as a hotspot of 0.785mm^2. A minimum threshold was set for the digital contrast to identify epithelial areas only, and to differentiate epithelium stained by pankeratin from the non-epithelial areas. By scanning at a 5x magnification, large pankeratin positive areas as well as debris, loose epithelial cells and macrophages were excluded by dedicated image analysis software. In the delineated invasive front and hotspot the software marked each independent stained area of 60 µm^2 to 500 µm^2. The cut-offs of 60 µm^2 and 500 µm^2 were set after careful evaluation of multiple EAC samples in the discovery cohort. Areas smaller than 60 µm^2 were interpreted as artifacts and were excluded from analysis. Areas greater than 500 µm2 did not qualify as tumor buds and were also excluded by the software. Next to the quantification of tumor buds per mm^2 at the entire invasive front as well as in the hotspot delineated by the pathologists, number of buds was calculated by automated selection with Visiomorph in a hotspot area of 0.785 mm^2 at the invasive front.

Immunohistochemistry

Next to H&E, TB was also assessed on pankeratin-desmin stained slides (pankeratin clone AE/AE3, dilution 1:800, Neomarkers, Fremont, CA, United States; desmin by De-R-11 ready to use, Ventana Medical Systems, Roche, Tuscon, AZ, USA). The slides were stained in an automated slide staining system (BenchMark Ultra, Ventana Medical Systems), in which the FFPE slides were deparaffinized, followed by heat-induced antigen retrieval using standard CC1 (Ventana Medical Systems) for 64 minutes. Subsequently samples were incubated with

pankeratin for 32 minutes, after which Protease1 was applied for eight minutes. Hereafter desmin was incubated for 32 minutes. Keratin was visualized by Ultraview Universal Dap (Ventana Medical Systems), while desmin by Ultraview Alkaline Phosphatase Red (Ventana Medical Systems) and counterstained with hematoxylin.

Ethics

The investigational protocol was approved by the medical ethical committee in the Erasmus MC and of all participating hospitals.

Study endpoints and statistical analysis

The primary endpoint of the study was presence or absence of LNM. In the resection specimen, at least 12 lymph-nodes were examined to establish the LNM status [173]. When less than 12 lymph-nodes were present in archival FFPE material of the resection specimens or when an endoscopic instead of radical resection was performed, LNM status was established based on clinical follow up of 5 years. Secondary endpoints were DFS and OS. DFS was defined as the time between surgery/endoscopic resection and the first clinical recurrence of disease, with clinical, radiological or pathological evidence of disease recurrence. OS was defined as time between surgery/endoscopic resection and patient all cause death. Patients lost to follow-up were censored at the time of the last visit to the outpatient clinics. The optimal cut-off for TB assessed with pankeratin was calculated by maximizing the Youden-index (supplemental Table 1 and supplemental Figure 1). The pN-stage was dichotomized in pN0 and a pN+ (pN1-3) group.

The interobserver agreement was calculated using the interclass correlation coefficient. Strength of agreement was categorized as follows: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; and 0.81–1.00, excellent. The best performing methods (e.g. highest intraclass correlation coefficient) were subsequently assessed using logistic regression models. Uni- and multivariable Cox proportional hazard models were applied to calculate the association between TB and survival. In multivariable analysis adjustments were made for all clinical and pathological factors which proved to be associated with LNM in a univariable analysis.

The analysis was performed using SPSS-software (version 22, SPSS IBM inc, Armonk, NY, USA). A cut-off of 0.05 was used to determine statistical significance.

RESULTS

Patient characteristics

In total 140 patients were included in this study, with a median age of 66.0 years (IQR: 58.4-73.0). Twenty patients were treated by endoscopic resection only. Thirty-four patients had an endoscopic resection followed by esophagectomy and 88 were primarily treated with a radical esophagectomy. Most EAC showed a moderate differentiation grade (n= 75), 19 EAC were well differentiated and 46 poorly differentiated. In 19.3% lympho-vascular invasion was found. Beside gender distribution, no other statistical differences were detected between the discovery and the validation cohort (see supplemental Table 2). Of all included tumors, 128 (91.4%) had more than five years of follow-up or more than 12 lymph nodes present in the resection specimen.

Interobserver variation in discovery cohort

The interclass correlation coefficient was separately calculated for H&E and pankeratin based assessments and was found to be at least good for all methods. The Ueno and Ohike methods showed the highest interobserver correlation (kappa=0.958 and 0.899 for H&E; 0.718 and 0.861 for pankeratin based method resp.; see supplemental Table 3). The Thies methods showed lower degree of agreement and were disregarded for further analysis (see supplemental Table 3).

Tumor budding correlates with LNM status and survival

Cut-offs for high vs. low TB for the H&E based Ueno and Ohike methods were chosen according to previous studies (see material and methods sections) [147,165]. Cut-offs for pankeratin based Ueno and Ohike methods were 14 and six buds respectively, according to the results of this study.

Next, all histopathological tumor characteristics were correlated with LNM status. In the discovery cohort, only Ohike was associated with LNM in the uni- and multivariable logistic regression analysis corrected for tumor differentiation and lympho-vascular invasion (both on H&E and pankeratin based methods, Table 1). In the validation cohort, Ohike based assessment on H&E slides remained significantly predictive for LNM in the multivariable analysis (OR 3.51), while pankeratin-based assessment lost significance.

Adding high TB (according to the Ohike H&E based method) to the other adverse pathological criteria for LNM status resulted in improved area under the curve (0.803 (95% CI 0.689-0.918) compared to 0.780 (95% CI 0.662-0.897)), see Supplemental Figure 2.

TABLE 1: Predictive value of tumor budding for the presence of lymph node metastasis using different scoring methods, including digital tumor bud count [164,172].

	Univariable Cox regression analysis					
	Discovery cohort (n=25)			Validation cohort (n=103)		
	OR	95% CI	p-value	OR	95% CI	p-value
H&E						
Ueno [164]	3.93	0.59-26.11	0.157	3.43	1.31-8.96	0.012
Ohike [164,172]	12.50	1.60-97.65	0.016	5.75	2.24-14.75	<0.001
Pankeratin staining						
Ueno	6.00	0.60-60.44	0.128	4.04	1.24-13.21	0.021
Ohike	30.00	2.58-348.77	0.007	6.03	2.10-17.35	0.001
DTBC						
TuB/mm2	15.60	1.48-164.38	0.022	2.73	0.94-7.97	0.066
TuB HS Comp	15.60	1.48-164.38	0.022	3.29	1.08-10.06	0.036
TuB HS Path	4.80	0.48-48.46	0.184	3.31	0.87-12.58	0.078
	Multivariable Cox regression analysis					
H&E						
Ueno [164]	3.23	0.38-27.29	0.281	1.82	0.56-5.95	0.321
Ohike [172]	21.03	1.30-341.07	0.032	3.51	1.05-11.68	0.041
Pankeratin staining						
Ueno	7.19	0.37-140.68	0.193	2.11	0.44-10.04	0.348
Ohike	22.77	1.65-314.79	0.020	2.56	0.69-9.45	0.158
DTBC						
TuB/mm2	13.53	0.95-193.26	0.055	1.06	0.26-4.44	0.933
TuB HS Aut	13.53	0.95-193.26	0.055	1.62	0.38-6.88	0.511
TuB HS Path	7.194	0.37-140.68	0.193	2.08	0.41-10.51	0.374

OR: Odds Ratio; CI; Confidence interval; DTBC: digital tumor bud count; TuB: Tumor Bud; HS Aut: Automated selection of hotspot; HS Path: Selection of hotspot by pathologist

Next, prognostic value of TB for survival was analyzed. Ueno and Ohike methods showed significant correlation with OS and DFS in the univariable analysis (Table 2). In a multivariable analysis only H&E based Ohike method remained significant for predicting OS and DFS (HR of 2.20 and HR 2.99).

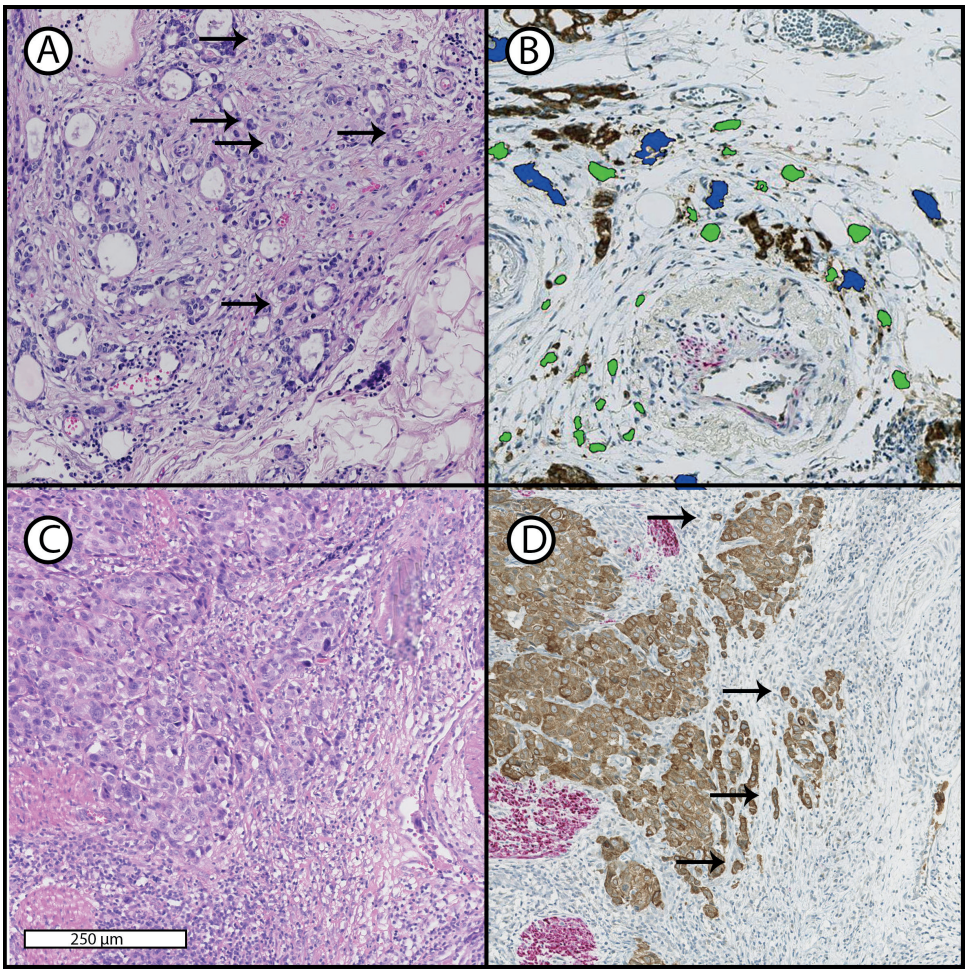


FIGURE 2: A: Tumor buds (TB; arrows) (H&E x 100 magnification). B: Immunohistochemistry (Pankeratin) used for Digital Tumor Bud Count. Software indicated the green areas as tumor buds and the blue areas (too large for tumor buds) as epithelium. C: EAC with extensive inflammatory infiltrate obscuring tumor buds, (H&E x 100 magnification). D: individual tumor buds (arrows). Double Immunohistochemical staining in which the epithelium is stained brown (pankeratin) and smooth muscle stained red (desmin), in which the tumor buds are readily identifiable. Compare H&E (A and C) and immunohistochemical stain (D).

Digital assessment of tumor budding (DTBC)

Besides the standard visual assessment by the pathologists as described above, the optimal cut-offs for the different DTBC methods were identified by optimizing the Youden index.

These were 17 tumor buds in 1mm^2 (TuB/mm2), 49 buds in a hotspot of 0.785 mm^2, as delineated by the pathologist (TuB/HS Path), and 25 buds in the automatically selected hotspot using software (TuB/HS Aut). TuB/mm2 and TuB/HS Aut were predictive for LNM in the univariable analysis in the discovery and validation cohort. However, the predictive value could not be confirmed in a multivariable analysis (see Table 1). Also, no correlation could be established with OS or DFS (see Table 2).

TABLE 2: Prognostic value of tumor budding for overall survival (OS) and disease free survival (DFS) using different scoring methods, including digital tumor bud count [164,172].

	Univariable Cox regression analysis					
	Overall Survival			Disease Free Survival		
	HR	95% CI	p-value	HR	95% CI	p-value
H&E						
Ueno[164]	1.77	1.00-3.11	0.049	2.57	1.02-6.48	0.046
Ohike [172]	2.62	1.51-4.56	0.001	3.71	1.54-8.96	0.004
Pankeratin staining						
Ueno	2.40	1.15-5.00	0.020	1.59	0.62-4.09	0.339
Ohike	1.78	0.97-3.26	0.064	3.30	1.28-8.52	0.014
DTBC						
TB/mm2	1.89	0.97-3.69	0.063	2.12	0.80-5.57	0.129
TB HS Comp	1.89	0.97-3.66	0.060	2.71	0.98-7.45	0.054
TB HS Path	1.67	0.79-3.50	0.179	2.69	0.79-9.19	0.114
	Multivariable Cox regression analysis					
H&E						
Ueno [164]	1.33	0.70-2.51	0.383	1.99	0.77-5.13	0.154
Ohike [172]	2.20	1.17-4.12	0.014	2.99	1.22-7.35	0.017
Pankeratin staining						
Ueno	1.51	0.67-3.39	0.324	1.20	0.46-3.18	0.711
Ohike	1.13	0.56-2.26	0.736	2.23	0.80-6.20	0.126
DTBC						
TuB/mm2	1.37	0.66-2.85	0.398	1.59	0.59-4.29	0.362
TuB HS Aut	1.45	0.71-2.98	0.306	2.12	0.76-5.97	0.153
TuB HS Path	1.28	0.59-2.78	0.538	2.01	0.57-7.04	0.276

HR: Hazard Ratio; CI; Confidence interval; DTBC: digital tumor bud count; TuB: Tumor Bud; HS Aut: Automated selection of hotspot; HS Path: Selection of hotspot by pathologist

DISCUSSION

In patients with pT1b EAC the risk for LNM is difficult to predict using current clinical and histological factors. [66,68,69,143,174]. In this study manual and digital TB methods were compared in relation to LNM and outcome in patients with pT1b EAC. High TB significantly increased the risk of LNM (OR 3.5) and tumor-related death (HR 2.2). Our results show clearly that TB is a potent and valuable biomarker for improved risk stratification in pT1b EAC.

TB has been considered as histological reflection of the epithelial-mesenchymal transition (EMT). EMT is a process in which neoplastic cells lose their epithelial characteristics, and gain mesenchymal features, increasing migratory possibilities [175-177]. In CRC, high TB is an informative marker of invasive potential and independent prognosticator for poor survival [144,162,163,167,178]. In CRC, TB is an established predictor of LNM status although the risk differs considerably between studies with OR ranging between 1.8-55.5 [149-152]. In a recent meta-analysis of pT1 CRC, risk for LNM was 6 fold higher in tumors with high TB [162]. However, since a clear and universally accepted standardized approach is still under debate, TB is not widely used yet for risk stratification in CRC [170].

Variety of methods has been applied for TB [162] with major differences in 1) definition of TuB; 2) definition of invasive front; 3) area of assessment: 4) cut-offs values for high vs. low TB. Given these major differences in TB approach, we aimed to compare different TB systems applied in gastrointestinal malignancies in earlier studies. Identification of buds using H&E staining might be difficult, particularly in cases with marked inflammation or prominent stromal cells. Since pankeratin immunohistochemistry enhances visualization of buds and was shown to improve agreement between pathologists[153-155,179,180], different TB methods were assessed both on H&E and pankeratin stained slides. In addition, TB was evaluated using digital image analysis. We found that reproducibility was good to excellent for all manual H&E based methods. The evaluation using pankeratin staining improved identification of tumor buds with median of 48 buds in 0.785mm2, compared to 30 buds on H&E. However, pankeratin staining did not improve agreement on high versus low TB compared to H&E based assessment. Also, pankeratin based assessment was not predictive of LNM status or outcome, also not after adjustment of the thresholds for high vs. low TB in immunohistochemically stained slides. The possible underlying problem could be that pankeratin highlights actually representing residual ductal structures or apoptotic tumor cells destroyed by inflammation. Although the precise explanation of the inferiority pankeratin based assessment compared to H&E as found in this study is lacking, our results are not surprising. Earlier studies showed that the predictive and prognostic value of TB was not increased by immunohistochemistry in various cancer types [168,181-183].

The results of the present study are in line with the results found in the single previous EAC study by Landau *et al.* employing both pT1a and pT1b tumors, in which TB was predictive for LNM (OR of 2.5) [147]. This study found also correlation between extensive TB and survival

in pT1 EAC (OS and DSF, HR of 3.3 and 3.2 resp.). In another publication assessing TB in heterogeneous EAC stages, subgroup analysis in pT1 EAC showed that TB was prognostic for survival [168]. However, interobserver variation was high in this study with kappa values ranging from 0.32 to 0.83, depending on the TB method as well as employment of pankeratin staining.

There are some limitations of the present study. Our cohort of patients was identified retrospectively, which could hamper the uniform classification of the data. To harmonize the pathological data all known histological parameters including differentiation grade, depth of invasion and lympho-vascular invasion were evaluated carefully by highly experienced pathologists.

Furthermore we included not only surgical but also endomucosal resection specimens. There was a variation in the applied surgical techniques and number of lymph nodes retrieved. Therefore, a minimal threshold of 12 lymph nodes was used to insure a representative LNM status. Furthermore in patients who were treated endoscopically, lymph nodes were not assessed. To circumvent this problem only patients with at least 5 years of follow-up were included in this study.

In conclusion, in patients with EAC and submucosal invasion (pT1b EAC), prognostication of LNM status is significantly improved by TB assessment. TB assessment by the H&E based Ohike method is reproducible and significantly associated with LNM and prognosis, independently from other histological tumor characteristics, such as depth of invasion, tumor grade and lympho-vascular invasion. Therefore TB evaluation should be implemented in the pathologically assessment of pT1b EAC to improve clinical management in these patients.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLE 1: Calculation of the optimal cut-off values for tumor budding on pankeratin stained slides for the standard pathological assessment using the Ueno method and Ohike method, as well as for the digital tumor budding count (DTBC).

	Positive if Greater Than or Equal To[a]	Sensitivity	Specificity	Youden index
Ueno	5.75	1.000	0.333	0.333
	9.50	1.000	0.389	0.389
	13.00	1.000	0.444	0.444
	14.75	**1.000**	**0.500**	**0.500**
	18.00	0.857	0.500	0.357
	21.50	0.857	0.556	0.413
	23.50	0.714	0.556	0.270
Ohike	3.25	0.857	0.500	0.357
	4.25	0.857	0.611	0.468
	5.25	0.857	0.722	0.579
	5.75	0.857	0.778	0.635
	6.50	**0.857**	**0.833**	**0.690**
	7.25	0.571	0.833	0.405
	7.75	0.286	0.833	0.119
DTBC TuB/mm2	10.50	0.857	0.444	0.302
	11.50	0.857	0.556	0.413
	13.50	0.857	0.611	0.468
	15.50	0.857	0.667	0.524
	17.00	**0.857**	**0.722**	**0.579**
	21.50	0.714	0.722	0.437
	28.00	0.571	0.722	0.294
DTBC TuB HS Aut	15.50	0.857	0.500	0.357
	20.50	0.857	0.667	0.524
	25.50	**0.857**	**0.722**	**0.579**
	27.50	0.714	0.722	0.437
	33.00	0.714	0.778	0.492
	38.50	0.571	0.778	0.349
DTBC TuB HS Path	37.00	0.714	0.611	0.325
	41.50	0.714	0.667	0.381
	49.50	**0.714**	**0.778**	**0.492**
	62.00	0.571	0.778	0.349
	69.50	0.571	0.833	0.405

TuB: Tumor Bud; HS Aut: Automated selection of hotspot;
HS Path: manual hotspot selection by pathologist

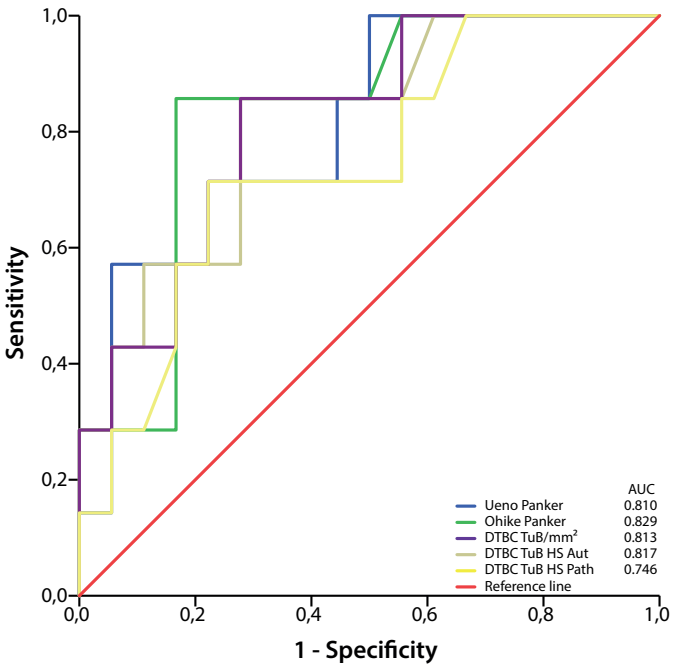
SUPPLEMENTAL TABLE 2: Patient characteristics for the entire cohort and specified for the discovery and validation cohort.

	Entire Cohort		Discovery cohort		Validation cohort		
	n	%	n	%	n	%	p-value
Age, Years (Median (IQR))	66.0 (58.4 – 73.0)		66.0 (56.0 –72.9)		65.6 (58.6 – 73.1)		0.998[¥]
Gender							
Male	121	86.4	19	70.4	102	90.3	0.007[§]
Female	19	13.6	8	29.6	11	9.7	
Treatment							
Endoscopic	20	14.3	4	14.8	16	14.2	0.250[§]
Endoscopic followed by surg	34	22.9	10	37.0	22	19.4	
Surgery	88	62.9	13	48.1	75	66.4	
Surgical Approach							
Transhiatal	99	70.7	20	90.9	79	85.9	0.904
Transthoracal	6	4.3	1	4.5	5	5.4	
Stomach resection	5	3.6	1	4.5	4	4.3	
Unknown	4	2.8	0	0	4	4.3	
Tumor location							
Esophagus	60	71.5	22	81.5	38	66.7	0.301[§]
GE-junction	18	12.9	3	11.1	15	26.3	
Cardia	4	54.8	1	3.7	3	5.3	
Tumor diameter							
≤ 2,0 cm	38	55.1	10	50.0	28	57.1	0.588[§]
> 2.0 com	31	44.9	10	50.0	21	42.9	
Tumor Grade							
Well	19	13.6	4	14.8	15	13.3	0.971[§]
Moderate	75	53.6	14	51.9	61	54.0	
Poor	46	32.9	9	33.3	37	32.7	
Lymph-Vasc Invasion							
No	113	80.7	22	81.5	91	80.5	0.910[§]
Yes	27	19.3	5	18.5	22	19.5	
Number of positive lymph nodes							
0 (pN0)	82	71.3	17	77.3	65	69.9	0.268[§]
1-2 (pN1)	21	18.2	2	9.0	19	20.4	
3-6 (pN2)	10	8.7	3	13.6	7	7.5	
>6 (pN3)	2	1.7	0	0.0	2	2.2	

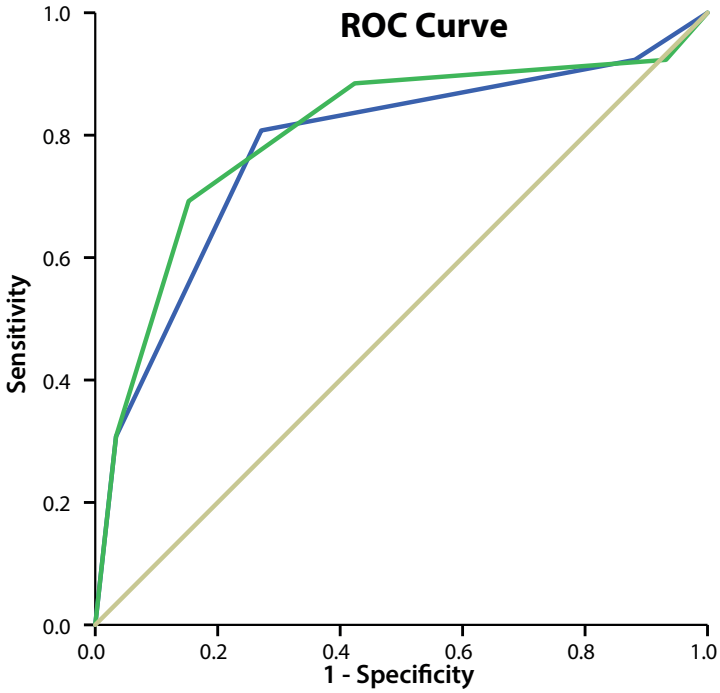
[§]: Pearson Chi-square; [¥]:Whitney U Test.

SUPPLEMENTAL TABLE 3: Interobserver agreement for different methods assessing tumor budding on a continues scale [164,168,172].

	Interclass coefficient	
		95% CI
Hematoxin & Eosin		
Ueno [164]	0.958	0.908-0.981
Ohike [172]	0.899	0.785-0.954
Thies 1 field [168]	0.912	0.811-0.961
Thies 10 fields [168]	0.734	0.486-0.873
Pankeratin staining		
Ueno	0.718	0.454-0.866
Ohike	0.861	0.526-0.949
Thies 1 field [168]	0.663	0.361-0.839
Thies 10 fields [168]	0.677	0.389-0.844

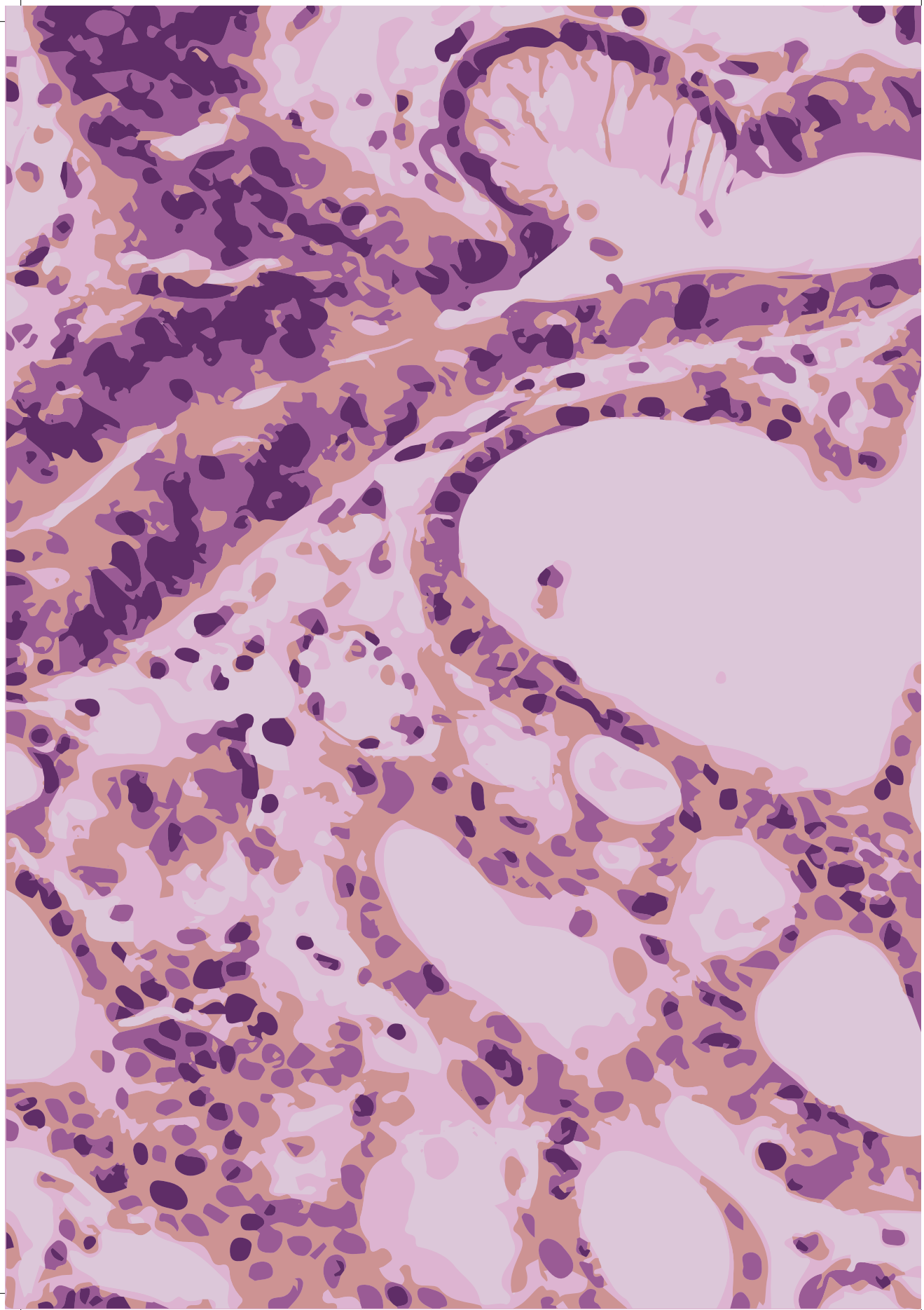


SUPPLEMENTAL FIGURE 1: Receiver Operating Characteristics (ROC) curve, based on the patients included in the discovery cohort. In the lower right corner the area under the curve (AUC) is indicated. Panker: assessed on the pankeratin stained slides; DTBC: Digital Tumor Budding Count; TuB: Tumor Bud; HS Aut: Automated selection of hotspot; HS Path: Selection of hotspot by pathologist



Adverse pathological critria	Area under the curve (95% CI)
without TB	0.780 (0.662 - 0.897)
with TB	0.803 (0.689 - 0.918)

SUPPLEMENTAL FIGURE 2: Receiver operating characteristics curve (ROC-curve) for the adverse pathological criteria for the prediction of LNM without tumor budding (TB) according to the Ohike H&E method and with TB, which shows an increase of the area under the curve from 0.780 to 0.803.



CHAPTER 6

Loss of SRY-box2 (SOX2) expression and its impact on survival of patients with esophageal adenocarcinoma

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ABSTRACT

Introduction: Esophageal adenocarcinoma (EAC) is a highly aggressive malignancy with poor survival, which is highly variable amongst patients with comparable conventional prognosticators. Therefore molecular biomarkers are urgently needed to improve the prediction of survival in these patients. SRY (sex determining region Y)-box 2, also known as SOX2, is a transcription factor involved in embryonal development of the gastrointestinal tract as well as in carcinogenesis. The purpose of this study was to see whether SOX2 expression is associated with survival in patients with EAC.

Methods: SOX2 was studied by immunohistochemistry in patients who had undergone potentially curative esophagectomy for adenocarcinoma. Protein expression of SOX2 was evaluated using tissue microarrays from resection specimens, and results were analysed in relation to the clinical data by Cox regression analysis. SOX2 was evaluated in two independent EAC cohorts (Rotterdam cohort and a multicentre UK cohort).

Results: Loss of SOX2 expression was independently predictive of adverse overall survival in the multivariable analysis, adjusted for known factors influencing survival, in both cohorts (Rotterdam cohort: hazard ratio (HR) 1.42, 95 per cent CI 1.07 to 1.89, $P = 0.016$; UK cohort: HR 1.54, 1.08 to 2.19, $P = 0.017$). When combined with clinicopathological staging, loss of SOX2 showed an increased effect in patients with pT1–2 tumors ($P = 0.010$) and node-negative EAC ($P = 0.038$), with incremental adverse effect on overall survival for stage I EAC with SOX2 loss (HR 3.18, 1.18 to 8.56; $P = 0.022$).

Conclusion: SOX2 is an independent prognostic factor for long-term survival in EAC, especially in patients with stage I EAC.

INTRODUCTION

Esophageal adenocarcinoma (EAC) is an aggressive cancer with a steadily increasing incidence[184,185]. The major risk factors for EAC are gastro-esophageal reflux[186], abdominal obesity[7] and Barrett's esophagus[32,119]. Patients with non-dysplastic Barrett's esophagus have a low rate of progression to EAC during surveillance (less than 1 per cent per year)[187], but most patients with EAC exhibit underlying Barrett's esophagus at the time of EAC diagnosis and are typically diagnosed at an advanced stage[188].

Although the addition of neoadjuvant therapy to primary surgical resection improves overall survival (OS) and disease-specific survival in patients with locally advanced tumors, the prognosis of most patients with advanced EAC, including those treated with curative intent, is dismal, with a 5-year survival rate of 47 per cent at best[78,189,190]. Postsurgical prognostication is currently based on tumor staging according to the AJCC staging system, supplemented by pathological criteria[75]. However, even after considering all known parameters including resection margin, nodal status, presence of vascular invasion, tumor grade and differentiation grade, the course of the disease remains variable[191-193]. Improving clinical decision-making is essential, especially in early EAC. In these patients numerous treatment modalities are available, depending on tumor characteristics, and the best treatment modality for the individual patient is still a matter of debate. One method for a better prognostication in early EAC is the use of biomarkers that might improve decision-making to determine the optimal treatment strategy.

Various signalling pathways essential for embryonal development are involved in cancer initiation and progression, including the sex determining region Y (SRY)-box2, also known as SOX2. SOX2 is a highly conserved gene coded on a single exon that plays a pivotal role in the maintenance of embryonic stem cells[194]. In the gastrointestinal tract it determines the formation and differentiation of esophageal and gastric epithelium during embryogenesis[58,195]. Besides its role in embryogenesis, SOX2 is involved in various malignancies including squamous cell carcinoma of the esophagus[196], gastric adenocarcinoma[61], prostate[197] and colorectal [198] cancer. SOX2 functions differ depending on the cell of origin, and both oncogenic and tumor suppressive mechanisms have been described. The *SOX2* gene may be amplified in squamous cell carcinoma of the esophagus and trachea, and acts as a lineage survival oncogene by promoting cell migration and proliferation[59,199]. Accordingly, upregulation of SOX2 is strongly associated with adverse outcomes in these patients[196]. In contrast, the opposite functions of SOX2 were shown in gastric adenocarcinoma, in which loss of SOX2 expression was correlated with worse prognosis. Phosphatase and tensin homologue (PTEN) has been proposed as a direct target of SOX2[61].

Little is known about the role of SOX2 in established EAC, although it has been shown in association with BE [95]. Non-dysplastic Barrett's esophagus exhibits mixed differentiation and

expresses gastric genes including *SOX2* and gastric mucins MUC5A and MUC6, as well as CDX2 as a marker of intestinal differentiation[11]. SOX2 was found in 98 per cent of the biopsies with non-dysplastic Barrett's esophagus, whereas only 72 per cent of low-grade dysplasia and 29 per cent of EAC samples demonstrated SOX2 expression[95]. Similar observations were detected for markers of intestinal differentiation[200,201]. It was concluded that *SOX2*, in parallel with the gastric mucins and intestinal genes, is gradually lost during progression of Barrett's esophagus to EAC[95]. SOX2 status has also been shown to be indicative of the pattern of response to neoadjuvant chemoradiotherapy in patients with EAC[202,203], and one small cohort study[204] suggested that SOX2 may have a prognostic effect for disease-free survival (DFS) in surgically treated patients with EAC.

The aim of the present study was to assess the role of SOX2 as a prognostic marker in patients with surgically treated EAC. As *SOX2* is lost during progression of Barrett's esophagus to EAC, it was hypothesized that this gene would have particular influence in stage I EAC.

METHODS

Patient selection

To reduce possible bias of neoadjuvant treatment that might influence SOX2 expression and interfere with OS, two historical EAC cohorts with a high proportion of patients who had surgical resection alone were used. Both the Rotterdam cohort and the UK multicentre cohort from the OCCAMS (Oesophageal Cancer Clinical And Molecular Stratification) study included patients who underwent esophagectomy with curative intent for pathologically confirmed adenocarcinoma of the esophagus or gastro-esophageal junction. Follow-up of all patients was performed in the respective clinical centres and only patients who were alive 1 month after surgery were included in the analysis. The Rotterdam cohort consisted of patients treated at the Department of Surgery at Erasmus Medical Centre, Rotterdam, between 1995 and 2006. The UK cohort comprised patients from six tertiary hospitals who were treated between 1992 and 2000.

Clinical and pathological data for both cohorts were collected, including tumor grade, pathological stage, anatomical location of the tumor divided in three types as described by Siewert[205], chemotherapy, age at surgery, co-morbidities and OS. The TNM system according to the UICC seventh edition[75] was used for pathological grading and staging. To ensure reliable classification, all tumors were reviewed by an expert gastrointestinal pathologist.

Tissue microarray

For the construction of a tissue microarray (TMA), formalin-fixed paraffin-embedded tissue from the resection specimens were retrieved from the archives at the Departments of Pathology of the participating institutions. For each tumor, three to six cores from multiple representative areas of EAC, as identified by a pathologist on haematoxylin and eosin-stained slides, were taken from the original paraffin blocks, including the central part and invasive front of the tumor[206,207].

SOX2 immunohistochemistry

The SOX2 immunohistochemical staining technique has been described extensively in previous publications[95,202]. In short, 5-µm sections were cut from the TMA, deparaffinized and rehydrated. Tissue from squamous cell carcinoma with clear positive staining for SOX2 was placed on each immunohistochemical slide of the TMAs as a positive control. Antigen retrieval was enhanced by heating in a Tris buffer. Endogenous peroxidase activity was blocked by incubating the slides in a solution of 0.3 per cent hydrogen peroxide in phosphate-buffered saline. Primary SOX2 antibody (AF2018, dilution 1 : 800, goat, polyclonal; R&D systems, Abingdon, UK) was applied for 22 h at 4°C. The secondary antibody was a biotinylated

horse antigoat IgG antibody (1 : 150, BA-4000; Vector Laboratories, Peterborough, UK). Visualization was achieved using the horseradish peroxidase avidin-biotin complex method and diaminobenzidine. Slides were counterstained with haematoxylin.

The immunohistochemically stained TMA slides from both cohorts were digitalized and scored independently by two investigators blinded to the clinical and pathological outcome. In case of disagreement, the cores were reviewed by both investigators simultaneously and consensus was achieved.

SOX2 was scored as positive or negative in each of the stained cores. As described previously[202], weak or strong nuclear expression of at least 50 per cent of the tumor cells was defined as positive, whereas nuclear expression in less than 50 per cent of tumor cells as well as cytoplasmic SOX2 expression were defined as negative. Because SOX2 expression might be heterogeneous in EAC, the overall expression in each tumor was calculated from all corresponding cores. Patients with fewer than three cores containing cells representative of the original EAC were excluded from analysis.

The optimal cut-off value of immunohistochemistry with SOX2 to predict survival was calculated by receiver operating characteristic (ROC) curve analysis in the Rotterdam cohort, using the area under the curve (AUC) as the performance measure (*Figure S1*, supporting information). Based on this evaluation, absence of SOX2 expression was defined by negative staining of SOX2 in more than 75 per cent of the cores; otherwise, SOX2 was considered to be present.

Ethics

The investigational protocols for both cohorts were approved by the relevant institutional review boards (MEC-12-469 and LREC 04/Q2006/2).

Statistical analysis

The primary endpoint in this study was 5-year OS, defined as time from surgery until death. Differences between the Rotterdam and UK cohorts were analysed using Student's *t* test for normal distributions and the Mann–Whitney *U* test for non-normal distributions of continuous variables, and χ^2 test for categorical variables. The equality of distribution was tested with Levene's test. Interobserver variation between the two investigators for scoring of SOX2 was calculated using Cohen's κ. Strength of agreement was categorized as follows: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; and 0.81–1.00, excellent.

Kaplan–Meier curves were used to plot the 5-year survival by SOX2 status and the distribution was analysed using the Logrank test. After imputation of missing variables using a linear regression model, univariable and multivariable Cox proportional hazard models were applied to estimate the independent association between SOX2 immunohistochemical

TABLE 1: Clinico-pathological characteristics, combined cohort and specified by Rotterdam and OCCAMS cohort.

	Combined (N=756)		Rotterdam (N=336)		OCCAMS (N=420)		
Characteristics	**N**	**%**	**N**	**%**	**N**	**%**	**P-value**
Age at surgery							
Median	65.4		64.7		66.0		**0.009**
Range	(33-90)		(33-90)		(33-88)		
Follow-up time, months							
Median	20.9		25.0		18.0		**0.004**
Range	(1-199)		(1-199)		(1-193)		
Sex							
Male	602	82.0%	293	87.2%	309	77.6%	**0.001**
Female	132	18.0%	43	12.8%	89	22.4%	
Siewert classification							
Type 1	460	69.7%	190	57.1%	270	82.6%	**<0.001**
Type 2	168	25.5%	126	37.8%	42	12.8%	
Type 3	32	4.8%	17	5.1%	15	4.6%	
Recurrence	182	54.2%	182	54.2%	NA		
Resection margin status							
pR0	396	71.0%	245	72.9%	151	68.0%	**0.212**
pR1	162	29.0%	91	27.1%	71	32.0%	
Histology grade							
Well	52	7.5%	26	7.7%	26	7.3%	**0.007**
Moderate	248	35.7%	139	41.4%	109	30.4%	
Poor	394	56.8%	171	50.9%	223	62.3%	
Pathologic T-stage							
pT1	79	11.2%	48	14.7%	31	8.2%	**0.001**
pT2	132	18.8%	59	18.0%	73	19.4%	
pT3	474	67.3%	218	66.7%	256	67.9%	
pT4	19	2.7%	2	0.6%	17	4.5%	
Pathologic N-stage							
pN0	245	35.9%	142	42.4%	103	29.6%	<**0.001**
pN1 or more	438	64.1%	193	57.6%	245	70.4%	
(Neo-)adjuvant treatment							
Yes	214	31.3%	68	20.2%	146	42.1%	**<0.001**
No	469	68.7%	268	79.8%	201	57.9%	
Alive after 60 months							
Yes	234	31.0%	106	31.5%	128	30.5%	0.752
No	522	69.0%	230	68.5%	292	69.5%	
SOX2							
Negative	436	66.1%	181	57.1%	255	74.3%	**<0.001**
Positive	224	33.9%	136	42.9%	88	25.7%	

expression and survival. In the multivariable analysis, adjustments were made for the clinical and pathological factors that were independently predictive in the univariable analysis. In addition, sensitivity analysis using a multivariable Cox proportional hazards model excluding all patients receiving chemotherapy or chemoradiotherapy with adjustment for clinical and pathological factors was performed to test the role of SOX2 in these patients. A multivariable analysis adjusted for all clinicopathological criteria that were independently predictive in the univariable analysis was performed, to estimate the independent association between SOX2 and survival for each of the stage groupings described in the TNM classification[75]. pN category was dichotomized as pN0 and pN+ (pN1–3) groups for the multivariable analysis. All analyses were performed using SPSS® version 22 software (IBM, Armonk, New York, USA). $P < 0.050$ was considered statistically significant.

RESULTS

Patient characteristics

The EAC cohort from Rotterdam consisted of 336 patients, whereas that from the OCCAMS study comprised 420 patients. Clinical characteristics of the patients from both cohorts are shown in *Table 1*. Patients from the OCCAMS cohort were older than those from Rotterdam (median 66.0 *versus* 64.7 years respectively; $P = 0.009$) and had a shorter median follow-up (18.0 *versus* 25.0 months; $P = 0.004$). A greater proportion of patients in the Rotterdam cohort had a tumor at the esophagogastric junction (Siewert type II) ($P < 0.001$), higher degree of differentiation ($P = 0.007$), earlier pT category ($P = 0.001$) and a greater likelihood of having pN0 disease ($P < 0.001$). Loss of SOX2 expression was more common in the OCCAMS cohort (74.3 per cent *versus* 57.1 per cent in the Rotterdam cohort; $P < 0.001$).

In the Rotterdam cohort, 68 patients (20.2 per cent) received neoadjuvant chemoradiotherapy (29) or chemotherapy (39). In the OCCAMS cohort, 146 patients (42.1 per cent) received neoadjuvant chemotherapy according to UK guidelines (*Table 1*).

Association between SOX2 expression and survival

The interobserver agreement for the assessment of SOX2 immunohistochemistry between the two observers was excellent ($\kappa = 0.92$, $P < 0.001$). After exclusion of patients with fewer than three representative cores available, TMAs from 537 of 756 patients were used in the final analysis of SOX2 immunohistochemistry (288 from the Rotterdam and 249 from the OCCAMS cohort). In total, SOX2 was positive in 186 cancers and negative in 351. Representative examples of SOX2 immunohistochemical expression patterns are shown in *Figure S2* (supporting information).

In the Rotterdam cohort, negative SOX2 was associated with a shorter median OS compared with positive SOX2 (19.5 *versus* 32.9 months respectively; $P = 0.001$). Median survival in the OCCAMS cohort was similar to that in the Rotterdam cohort (15.0 and 26.0 months for negative and positive SOX2 respectively; $P = 0.014$) (*Table S1*, supporting information). Corresponding Kaplan–Meier curves for the individual cohorts and the combined group are depicted in *Figure 1*.

SOX2 expression did not correlate with location of the tumor. In Siewert type I EAC, 32.9 per cent of the tumors showed loss of SOX2, whereas in Siewert type II and III loss of SOX2 was found in 40.3 and 32.3 per cent of tumors respectively ($P = 0.260$).

Univariable analysis showed a hazard ratio (HR) for death in patients with SOX2 loss of 1.54 (95 per cent CI 1.16 to 2.04; $P = 0.003$) for the Rotterdam cohort, 1.58 (1.12 to 2.22; $P = 0.009$) for the OCCAMS cohort and 1.55 (1.25 to 1.93; $P < 0.001$) for the combined cohort (*Table S2*, supporting information).

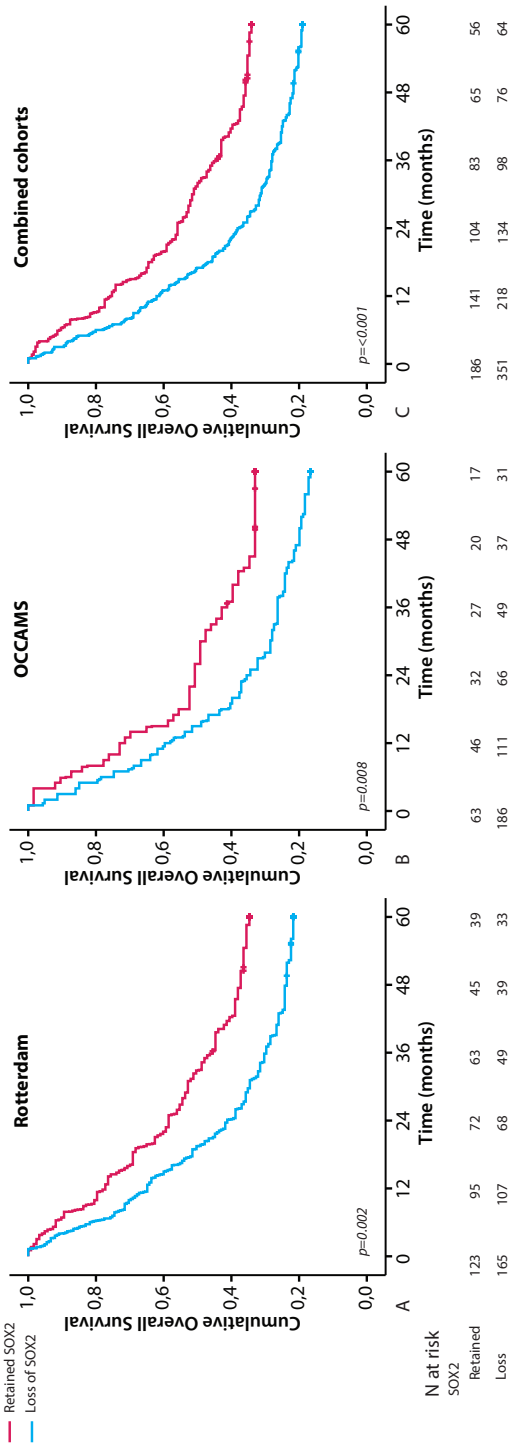

FIGURE 1: Expression of SOX2 is prognostic for overall survival; Rotterdam cohort (A), OCCAMS cohort (B) and combined cohort (C) (p-values are indicated in the left lower corner of each graph).

Multivariable regression analysis to test the independent value of SOX2 in relation to other clinical parameters showed that SOX2 remained significant for OS in both individual cohorts as well as in the combined cohort (HR 1.42, 95 per cent CI 1.14 to 1.77; $P = 0.002$) (*Table 2*).
Information on DFS was available only for the Rotterdam cohort; SOX2 was independently predictive of disease recurrence (HR 1.37, 95 per cent CI 1.01 to 1.86; $P = 0.045$) (*Table S3* and *Figure S3*, supporting information).
In chemotherapy-naive patients, SOX2 loss was confirmed as a statistically significant prognostic indicator of worse OS in both univariable and multivariable analysis (*Table 3*; *Table S4*, supporting information). When the prognostic value of SOX2 in chemotherapy-naive patients was examined in relation to clinicopathological staging, SOX2 showed separation into prognostic groups for pT1–2 tumors (HR 2.36, 95 per cent CI 1.23 to 4.51; $P = 0.010$) but not for pT3–4 tumors (*Figure 2a*; *Table S5* and *S6*, supporting information). Patients with pT1 EAC and loss of SOX2 had a trend towards being pN+ ($P = 0.070$) (*Table S7*, supporting information), whereas for pT2–4 tumors there was no correlation between SOX2 and nodal status.
When combining SOX2 and pN category, a significant separation into prognostic groups was detected for patients with pN0 disease (HR 1.71, 95 per cent CI 1.03 to 2.85; $P = 0.038$), whereas for pN1–3 no effect of SOX2 was seen (*Figure 2b*; *Table S8*, supporting information).
Based on the findings for pT and pN status, Kaplan–Meier curves were constructed for the effects of SOX2 for each TNM stage. Only in stage I disease was SOX2 loss associated with an increased HR for death (HR 3.18, 95 per cent CI 1.18 to 8.56; $P = 0.022$) (*Figure 2c*; *Table S9*, supporting information).
During follow-up, 289 chemotherapy-naive patients died within 5 years of surgery, of whom 194 showed loss of SOX2. The sensitivity of SOX2 for the prediction of death within 5 years in these patients was 67.1 per cent and the specificity 51.1 per cent. Of the 64 chemotherapy-naive patients with stage I disease, 19 died within 5 years, of whom 13 showed loss of SOX2. The sensitivity of SOX2 for prediction of death in chemotherapy-naive patients with stage I disease was 68 per cent and the specificity 62 per cent. Positive and negative predictive values and AUC for all patients, chemotherapy-naive patients and patients with chemotherapy-naive stage I EAC are shown in *Table S10* (supporting information).

TABLE 2: Multivariate survival analysis, for all patients (specified in Rotterdam and OCCAMS cohort). Positive SOX2 expression was used as reference. For the corresponding univariate analysis see supplemental data.

	Combined (N=402)			Rotterdam (N=287)			OCCAMS (N=115)		
Characteristic	**HR**	**95% CI**	**P- value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Multivariate analysis in entire cohort									
Age at surgery (per year increase)	1.02	1.01-1.03	0.002	Na	Na	Na	Na	Na	Na
pT-stage (pT1 ref)									
pT2	1.59	0.93-2.72	0.084	1.12	0.55-2.24	0.759	2.45	0.99-6.07	0.053
pT3-4	2.96	1.80-4.91	<0.001	2.60	1.40-4.84	0.003	3.58	1.46-8.80	0.005
pN-stage (pN0 ref)									
pN1 or more	1.68	1.15-2.46	0.011	1.57	1.14-2.17	0.006	1.89	0.81-4.45	0.121
Resection margin status (pR0 ref)									
pR1	1.15	0.88-1.50	0.313	1.27	0.93-1.75	0.133	1.01	0.66-1.57	0.949
Histology grade (well/moderate ref)									
Poor	1.57	1.25-1.97	<0.001	1.52	1.13-2.05	0.006	1.44	1.04-2.00	0.028
(Neo-)adjuvant treatment (Yes ref)									
No	Na	Na	Na	1.74	1.14-2.67	0.011	Na	Na	Na
SOX2 (positive ref)									
Negative	1.42	1.14-1.77	0.002	1.42	1.07-1.89	0.016	1.54	1.08-2.19	0.017

HR=Hazard ratio, CI=Confidence Interval, Na=Not available

TABLE 3: Multivariate survival analysis for SOX2-expression in chemotherapy naïve patients. Positive SOX2 expression is used as reference. For the corresponding univariate analysis see supplemental data.

	Combined (N=297)			Rotterdam (N=241)			OCCAMS (N=56)		
Characteristic	**HR**	**95% CI**	**P- value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Multivariate analysis									
Age at surgery (per year increase)	1.02	1.01-1.03	0.002	Na	Na	Na	Na	Na	Na
pT-stage (pT1 ref)									
pT2	1.88	0.96-3.68	0.065	1.40	0.64-3.09	0.400	3.11	0.77-12.52	0.110
pT3-4	3.99	2.13-7.48	<0.001	3.48	1.70-7.09	0.001	4.61	1.16-18.33	0.030
pN-stage (pN0 ref)									
pN1 or more	1.61	1.15-2.25	0.006	1.47	1.04-2.07	0.028	2.12	1.04-4.29	0.039
Resection margin status (pR0 ref)									
pR1	1.17	0.89-1.54	0.270	1.27	0.91-1.76	0.162	1.14	0.67-1.94	0.63
Histology grade (well/moderate ref)									
Poor	1.51	1.16-1.97	0.003	1.47	1.07-2.03	0.017	Na	Na	Na
SOX2 (positive ref)									
Negative	1.35	1.04-1.75	0.026	1.40	1.03-1.91	0.030	1.53	0.95-2.47	0.081

HR=Hazard ratio, CI=Confidence Interval.

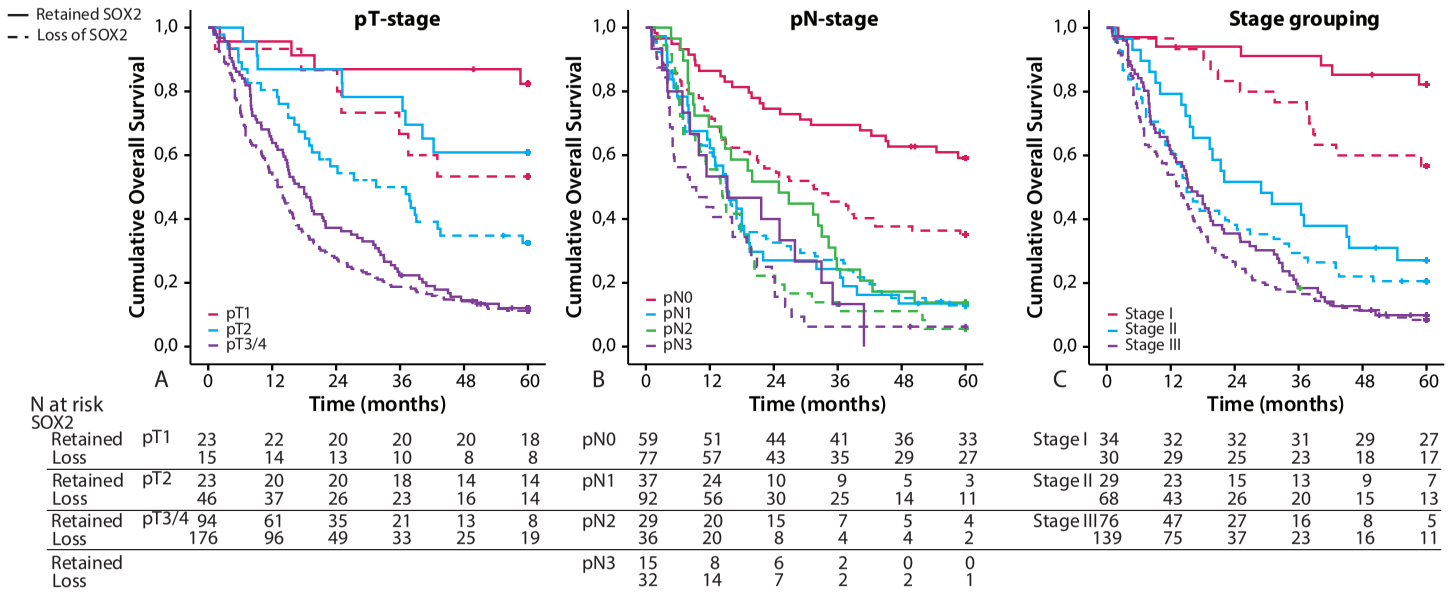

FIGURE 2: SOX2 expression in combination to clinico-pathological staging (a: pT-stage, b: pN-stage and c: stage groupings) segregates chemotherapy naïve patients, into prognostic groups in early EAC (pT1/pT2 tumors, pN-tumors and stage I tumors, p<0.05).

DISCUSSION

SOX2 immunohistochemistry adds prognostic information in patients with EAC. SOX2 loss was predictive of an adverse outcome in two independent cohorts (Rotterdam and OCCAMS), with a significant incremental adverse effect for OS, especially for patients with pN0 and stage I EAC.

Previous studies that attempted to identify clinically applicable predictive biomarkers for treatment response or overall prognosis have often been underpowered[83] or included heterogeneous patient populations with squamous cell carcinoma and adenocarcinoma[208]. Biomarker analysis can also be hampered by different neoadjuvant treatments in advanced EAC, making comparisons between studies difficult[209]. Large collaborative projects using standardized methodology are required to generate a clinically useful approach. Using this strategy, a three-gene immunohistochemical panel was shown to be useful in a previous large multicentre study[210]. Combining TNM staging with this immunohistochemical panel of epidermal growth factor receptor (EGFR), tripartite motif-containing 44 (TRIM44) and sirtuin 2 (SIRT2) allowed segregation of patients with stage II and III disease into distinct prognostic groups, whereas the effect for stage I was minimal[210]. This is different from the SOX2 findings reported here.

Little is yet known about the role of SOX2 in EAC. In Barrett's esophagus, which exhibits mixed intestinal and gastric differentiation, SOX2 is detected in most patients, whereas during the progression to EAC downregulation of gastric and intestinal gene expression, including SOX2, occurs[95,200,201]. In advanced EAC, retained expression of SOX2 has previously been related to resistance to neoadjuvant chemoradiotherapy in patients treated according to the CROSS (ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study) regimen[202,203]. An earlier small Dutch study of 94 patients with surgically treated EAC also suggested SOX2 loss to be a predictor of reduced DFS, although it was underpowered to establish the incremental value of SOX2 in OS[204]. The present study focused on surgically treated EAC and not only confirmed the prognostic value of SOX2 for DFS (HR 1.37; $P = 0.045$), but also showed that SOX2 loss predicted adverse OS in patients with EAC. Importantly, SOX2 status was independent of all clinical and histological parameters known to influence survival, including neoadjuvant treatment.

Patients with stage I EAC generally have a good prognosis with 5-year survival rates of 87.7 and 73.3 per cent for stages Ia and Ib respectively[81]. Although patients with pT1a disease can be treated by endoscopic resection or surgery alone, treatment of those with pT1b disease is more controversial owing to the risk of lymph node metastasis. An optimal treatment strategy for these patients is widely debated[67]. The benefits of neoadjuvant therapy, for instance, are unclear[211]. In the present study a worse OS in chemotherapy-naive patients

with stage I EAC was associated with loss of SOX2 (HR 3.18; $P = 0.022$). The results suggest that SOX2 might predict lymph node metastasis in pT1 EAC, although further studies are needed to confirm this.

The role of SOX2 in the pathogenesis of EAC is poorly understood. Significant association of retained SOX2 expression and favourable survival could be explained by SOX2 function as a tumor suppressor gene, similar to the findings in gastric carcinoma. Lower mitotic rate, increased apoptosis, and reduced invasion and dissemination were detected in patients with gastric cancer with retained SOX2 expression, compared with findings in those with SOX2 loss[60,62,212]. In line with its tumor suppressive role, several downstream targets of SOX2 were identified in gastric cancer, including cyclin D1 (CCND1), phosphorylated retinoblastoma 1 (pRB1), cyclin-dependent kinase inhibitor 1B (CDKN1B), as well as PTEN and phosphorylated protein kinase B (pAKT)[60,62,213]. Given the lineage-specific SOX2 function in formation of the stomach and esophagus during embryogenesis, the role of SOX2 in EAC might be similar to that seen in gastric cancer.

The present study has some limitations, including its retrospective design and the small number of patients with stage I tumors. The expression of SOX2 was assessed in TMAs constructed from resection specimens and not in preoperative biopsies of patients with EAC, which may also be important. Validation of these results in a prospective study, and on pretreatment tumor material as well as resection specimens, still needs to be undertaken. At the same time, SOX2 detection in this study was performed by standardized immunohistochemistry, which is readily reproducible, and although interpretation may be subjective there was excellent interobserver agreement ($\kappa = 0.92$), indicating that accurate classification of SOX2 pattern is possible.

Immunohistochemical detection of SOX2 provided useful prognostic information in patients with EAC, independent of clinical parameters. Use of this marker in addition to current staging systems could be of particular relevance in selected populations of patients with node-negative tumors and those with stage I disease. The precise biological role of SOX2 in EAC requires further elucidation.

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLE 1: Median (inter quartile range) overall survival (in months) according to SOX2 expression for the entire patient population and specified by Rotterdam and OCCAMS cohort .

	Combined (N=539)	Rotterdam (N=288)	OCCAMS (N=251)
SOX2			
Positive	31.7 (12.5-60.0)	32.9 (14.5-60.0)	26.0 (10.0-60.0)
Negative	17.0 (6.9-42.0)	19.5 (7.1-43.3)	15.0 (6.0-38.2)
Overall	19.7 (8.0-51.1)	22.3 (9.3-60.0)	16.0 (7.0-44.2)

SUPPLEMENTAL TABLE 2: Univariate survival analysis for all patients included in the study, specified for the combined cohort, Rotterdam cohort and OCCAMS cohort.

	Combined (N=537)			Rotterdam (N=288)			OCCAMS (N=249)		
Characteristic	HR	95% CI	P- value	HR	95% CI	P-value	HR	95% CI	P-value
Univariate analysis									
Age at surgery (per year increase)	1.01	1.00-1.02	0.011	1.01	0.99-1.02	0.262	1.02	1.00-1.03	0.014
Sex (Female ref)									
Male	0.99	0.77-1.27	0.920	1.12	0.76-1.66	0.577	0.91	0.65-1.27	0.575
pT-stage (pT1 ref)									
pT2	1.99	1.14-3.45	0.015	1.44	0.72-2.87	0.300	2.93	1.14-7.56	0.026
pT3-4	4.42	2.72-7.19	<0.001	4.09	2.27-7.37	<0.001	5.21	2.19-12.39	<0.001
pN-stage (N0 ref)									
pN1	1.99	1.45-2.73	<0.001	1.71	1.16-2.52	0.007	2.29	1.29-4.07	0.008
pN2	2.65	1.64-4.28	0.001	2.50	1.73-3.61	<0.001	2.87	0.66-12.61	0.135
pN3	4.48	2.99-6.70	<0.001	3.90	2.63-5.78	<0.001	12.24	2.12-70.69	0.009
pN-stage (pN0 ref)									
pN1 or more	2.33	1.58-3.44	<0.001	2.40	1.77-3.26	<0.001	2.27	0.98-5.25	0.054
Resection margin status (pR0 ref)									
pR1	1.83	1.44-2.32	<0.001	2.39	1.80-3.18	<0.001	1.42	0.97-2.06	0.070
Histology grade (well/moderate ref)									
Poor	1.76	1.41-2.20	<0.001	2.05	1.55-2.71	<0.001	1.46	1.02-2.10	0.039
(Neo-)adjuvant treatment (Yes ref)									
No	1.20	0.85-1.70	0.279	1.75	1.15-2.66	0.009	0.97	0.58-1.62	0.889
SOX2 (positive ref)									
Negative	1.55	1.25-1.93	<0.001	1.54	1.16-2.04	0.003	1.58	1.12-2.22	0.009

HR=Hazard ratio, CI=Confidence Interval, Na=Not available

SUPPLEMENTAL TABLE 3: Univariate and multivariate survival analysis for disease free survival in the Rotterdam cohort.

Characteristic	Rotterdam cohort (N=288)		
	HR	95% CI	P- value
Univariate analysis			
Age at surgery (per year increase)	1.00	0.98-1.01	0.503
Sex (Female ref)			
Male	1.31	0.87-1.98	0.194
pT-stage (pT1 ref)			
pT2	1.45	0.68-3.07	0.337
pT3-4	4.27	2.24-8.13	<0.001
pN-stage (N0 ref)			
pN1	2.18	1.42-3.34	<0.001
pN2	2.95	1.95-4.45	<0.001
pN3	5.00	3.24-7.71	<0.001
pN-stage (pN0 ref)			
pN1 or more	2.98	2.10-4.21	<0.001
Resection margin status (pR0 ref)			
pR1	2.47	1.82-3.35	<0.001
Histology grade (well/moderate ref)			
Poor	2.16	1.60-2.93	<0.001
(Neo-)adjuvant treatment (Yes ref)			
No	1.62	1.04-2.54	0.035
SOX2 (positive ref)			
Negative	1.49	1.10-2.01	0.010
Multivariate analysis			
pT-stage (pT1 ref)			
pT2	1.08	0.51-2.32	0.837
pT3-4	2.44	1.23-4.82	0.010
pN-stage (pN0 ref)			
pN1 or more	1.95	1.35-2.82	<0.001
Resection margin status (pR0 ref)			
pR1	1.31	0.93-1.85	0.119
Histology grade (well/moderate ref)			
Poor	1.48	1.07-2.05	0.018
(Neo-)adjuvant treatment (Yes ref)			
No	1.60	1.02-2.53	0.042
SOX2 (positive ref)			
Negative	1.37	1.01-1.86	0.045

HR=Hazard ratio, CI=Confidence Interval

SUPPLEMENTAL TABLE 4: Univariate survival analysis in chemotherapy naïve patients, specified for the combined cohort, Rotterdam cohort and OCCAMS cohort.

	Combined (N=377)			Rotterdam (N=241)			OCCAMS (N=136)		
Characteristic	**HR**	**95% CI**	**P- value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Univariate analysis									
Age at surgery (per year increase)	1.01	1.00-1.03	0.026	1.01	1.00-1.03	0.195	1.02	1.00-1.04	0.057
Sex (Female ref)									
Male	0.91	0.68-1.21	0.527	0.95	0.63-1.43	0.814	0.88	0.59-1.31	0.521
pT-stage (pT1 ref)									
pT2	2.45	1.26-4.78	0.009	1.92	0.88-4.17	0.100	3.90	0.95-16.01	0.059
pT3-4	6.11	3.36-11.14	<0.001	5.72	2.91-11.26	<0.001	7.76	2.07-29.17	0.002
pN-stage (N0 ref)									
pN1	2.16	1.55-3.02	<0.001	1.93	1.28-2.92	0.002	2.48	1.36-4.52	0.005
pN2	2.50	1.63-3.83	<0.001	2.37	1.60-3.50	<0.001	2.79	0.78-9.97	0.102
pN3	3.83	2.56-5.74	<0.001	3.39	2.23-5.16	<0.001	12.63	2.14-74.43	0.008
pN-stage (pN0 ref)									
pN1 or more	2.53	1.86-3.43	<0.001	2.41	1.74-3.33	<0.001	2.74	1.44-5.22	0.004
Resection margin status (pR0 ref)									
pR1	2.05	1.55-2.71	<0.001	2.31	1.71-3.12	<0.001	1.74	1.05-2.89	0.033
Histology grade (well/moderate ref)									
Poor	1.87	1.42-2.47	<0.001	2.05	1.52-2.76	<0.001	1.57	0.88-2.80	0.117
SOX2 (positive ref)									
Negative	1.60	1.25-2.05	<0.001	1.57	1.16-2.11	0.003	1.67	1.06-2.63	0.027

HR=Hazard ratio, CI=Confidence Interval, Na=not available.

SUPPLEMENTAL TABLE 5A: Survival analysis in chemotherapy naïve patients. Univariate and multivariate survival analysis for the combination of pT1 and pT2 tumors and pT3 and pT4 tumors.

	pT1/pT2 (N=107)			pT3/pT4 (N=270)		
Characteristic	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Univariate analysis						
Age at surgery (per year increase)	1.01	0.98-1.05	0.225	1.02	1.01-1.03	0.002
Sex (female ref)						
Male	0.70	0.32-1.51	0.697	1.02	0.74-1.39	0.917
pN-stage (pN0 ref)						
pN1	3.90	1.99-7.67	<0.001	1.30	0.89-1.88	0.168
pN2	4.92	1.93-12.53	0.001	1.42	0.89-2.27	0.137
pN3	9.82	3.11-31.01	<0.001	2.01	1.30-3.11	0.002
pN-stage (pN0 ref)						
pN1 or more	4.30	2.22-8.30	<0.001	1.48	1.04-2.10	0.029
Resection margin status (pR0 ref)						
pR1	1.86	0.43-8.12	0.401	1.35	1.02-1.80	0.040
Histology grade (well/moderate ref)						
Poor	2.83	1.50-5.36	0.002	1.39	1.05-1.83	0.021
SOX2 (positive expression ref)						
Negative	3.08	1.66-5.78	<0.001	1.27	0.97-1.66	0.084
Multivariate analysis						
pN-stage (pN0 ref)						
pN1 or more	3.04	1.54-6.00	0.002	Na	Na	Na
Histology grade (well/moderate ref)						
Poor	1.72	0.88-3.35	0.110	Na	Na	Na
SOX2 (positive ref)						
Negative	2.36	1.23-4.51	0.010	Na	Na	Na

HR=Hazard ratio, CI=Confidence Interval, Na=not available.

SUPPLEMENTAL TABLE 5B: Survival analysis in chemotherapy naïve patients. Univariate and multivariate survival analysis for pT1 and pT2 tumors separately.

	pT1 (N=38)			pT2 (N=69)		
Characteristic	**HR**	**95% CI**	**P- value**	**HR**	**95% CI**	**P-value**
Univariate analysis						
Age at surgery (per year increase)	1.02	0.95-1.10	0.567	1.01	0.97-1.05	0.662
Sex (Female ref)						
Male	0.54	0.07-4.32	0.558	0.58	0.25-1.36	0.206
pN-stage (pN0 ref)						
pN1	7.45	1.76-31.57	0.006	2.68	1.24-5.79	0.013
pN2	10.52	1.74-63.66	0.010	3.29	1.09-9.97	0.035
pN3	10.85	1.08-108.97	0.043	7.66	1.93-30.36	0.004
pN-stage (pN0 ref)						
pN1 or more	8.55	2.50-29.16	0.001	2.87	1.33-6.19	0.008
Resection margin status (pR0 ref)						
pR1	Na	Na	Na	3.39	0.77-15.00	0.106
Histology grade (well/moderate ref)						
Poor	11.74	2.82-48.94	0.001	1.73	0.85-3.53	0.132
SOX2 (positive expression ref)						
Negative	3.46	1.01-11.85	0.048	2.48	1.17-5.28	0.018
Multivariate analysis						
pN-stage (pN0 ref)						
pN1 or more	1.88	0.30-11.84	0.502	2.86	1.32-6.18	0.009
Histology grade (well/moderate ref)						
Poor	7.41	1.27-43.14	0.026	Na	Na	Na
SOX2 (positive expression ref)						
Negative	2.20	0.50-9.59	0.295	2.42	1.12-5.22	0.025

HR=Hazard ratio, CI=Confidence Interval, Na=Not available

SUPPLEMENTAL TABLE 6: SOX2 in relation to lymph-node status in chemotherapy naïve patients with pT1 EAC.

	Chemo-naïve pT1 patients (N=38)		
	pN0	pN+	P-value
SOX2			
Positive	21 (91.3%)	2 (8.7%)	
Negative	10 (66.7%)	5 (33.3%)	0.070

SUPPLEMENTAL TABLE 7: Univariate and multivariate survival analysis in chemotherapy naïve patients for pN0 and pN+ tumors (pN+ is combination of pN1, pN2 and pN3).

	pN0 (N=136)			pN+ (N=241)		
Characteristic	**HR**	**95% CI**	**P- value**	**HR**	**95% CI**	**P-value**
Univariate analysis						
Age at surgery (per year increase)	1.02	0.99-1.04	0.242	1.01	1.00-1.03	0.069
Sex (female ref)						
Male	0.84	0.47-1.50	0.549	1.06	0.75-1.50	0.753
pT-stage (pT1 ref)						
pT2	2.73	0.98-7.64	0.056	1.02	0.42-2.49	0.963
pT3/4	8.61	3.45-21.44	<0.001	1.73	0.80-3.93	0.159
Resection margin status (pR0 ref)						
pR1	2.79	1.66-4.69	<0.001	1.47	1.10-1.95	0.009
Histology grade (well/moderate ref)						
Poor	2.39	1.50-3.80	<0.001	1.24	0.89-1.74	0.198
SOX2 (positive ref)						
Negative	2.25	1.38-3.68	0.001	1.23	0.91-1.65	0.178
Multivariate analysis						
pT-stage (pT1 ref)						
pT2	2.15	0.75-6.19	0.155	Na	Na	Na
pT3-4	5.73	2.20-14.90	<0.001	Na	Na	Na
Resection margin status (pR0 ref)						
pR1	1.18	0.66-2.11	0.578	Na	Na	Na
Histology grade (well/moderate ref)						
Poor	1.91	1.16-3.25	0.017	Na	Na	Na
SOX2 (positive ref)						
Negative	1.71	1.03-2.84	0.038	Na	Na	Na

HR=Hazard ratio, CI=Confidence Interval.

SUPPLEMENTAL TABLE 8: Univariate and multivariate survival analysis chemotherapy naïve patients in stage I (combination of stage Ia and stage Ib), stage II (combination of stage IIa and stage IIb) and stage III (combination of stage IIIa, stage IIIb and stage IIIc).

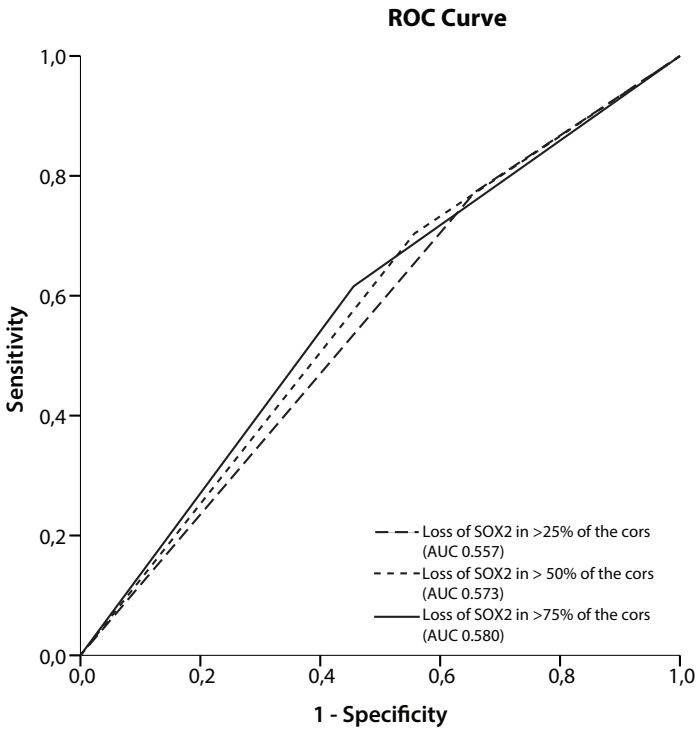
	Stage I (N=64)			Stage II (N=97)			Stage III (N=216)		
Characteristic	**HR**	**95% CI**	**P- value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Univariate analysis									
Age at surgery (per year increase)	1.01	0.95-1.07	0.782	1.02	0.99-1.05	0.095	1.02	1.00-1.03	0.030
Sex (female ref)									
Male	1.09	0.39-3.11	0.867	0.90	0.49-1.67	0.745	1.04	0.73-1.48	0.829
pT-stage (pT1 ref)									
pT2	2.73	0.99-7.73	0.052	1.07	0.31-3.70	0.913	0.77	0.21-2.84	0.6.89
pT3-4	Na	Na	Na	1.34	0.42-4.28	0.627	1.30	0.41-4.09	0.654
pN-stage (pN0 ref)									
pN1	Na	Na	Na	0.79	0.47-1.35	0.394	1.89	0.22-16.33	0.538
pN2	Na	Na	Na	Na	Na	Na	2.06	0.25-17.29	0.480
pN3	Na	Na	Na	Na	Na	Na	3.16	0.37-2745	0.275
pN-stage (pN0 ref)									
pN1 or more	Na	Na	Na	0.85	0.50-1.43	0.540	3.76	0.02-854.95	0.609
Resection margin status (pR0 ref)									
pR1	2.12	0.25-18.16	0.492	1.50	0.90-2.49	0.120	1.30	0.94-1.81	0.114
Histology grade (well/moderate ref)									
Poor	7.70	2.68-22.17	<0.001	1.22	0.76-1.97	0.408	1.23	0.90-1.67	0.191
SOX2 (positive ref)									
Negative	3.20	1.19-8.58	0.021	1.31	0.79-2.16	0.300	1.17	0.86-1.59	0.307
Multivariate analysis									
Histology grade (well/moderate ref)									
Poor	7.70	2.65-22.35	<0.001	Na	Na	Na	Na	Na	Na
SOX2 (positive ref)									
Negative	3.18	1.18-8.56	0.022	Na	Na	Na	Na	Na	Na

HR=Hazard ratio, CI=Confidence Interval, Na=Not available

SUPPLEMENTAL TABLE 9: Sensitivity , specificity, positive and negative predictive value, prevalence, accuracy and AUC of SOX2 loss to predict 5-year survival in all patients, chemotherapy naïve patients and stage I EAC.

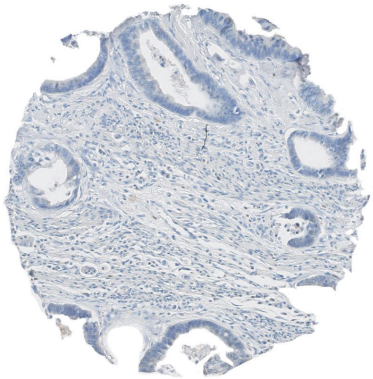
	Sens.	Spec.	PPV	NPV	Prev.	Accuracy	AUC	95% CI AUC
All patients	70.0%	48.9%	80.9%	34.4%	75.6%	64.8%	0.59	0.537-0.651
Chemotherapy-naïve patients	67.1%	51.1%	81.9%	32.1%	76.7%	63.4%	0.59	0.522-0.660
Stage I chemotherapy-naïve patients	68.4%	62.2%	43.3%	82.4%	29.7%	64.1%	0.65	0.506-0.800

Sens: sensitivity, Spec: specificity, PPV: positive predictive value, NPV: negative predictive value, Prev: prevalence, AUC: area under the curve, CI: confidence interval

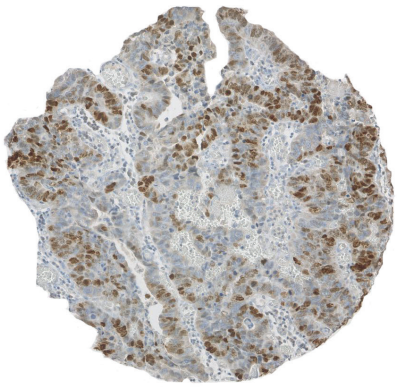


SUPPLEMENTAL FIGURE 1: ROC-curves according to the percentages of SOX2 loss in Rotterdam cohort.

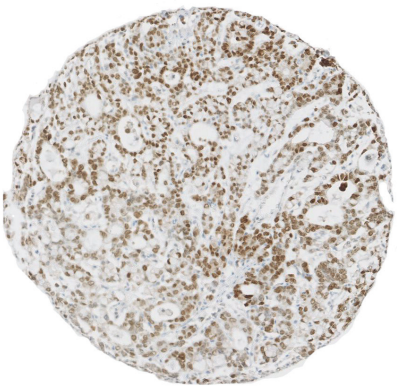
A: Loss of SOX2 expression



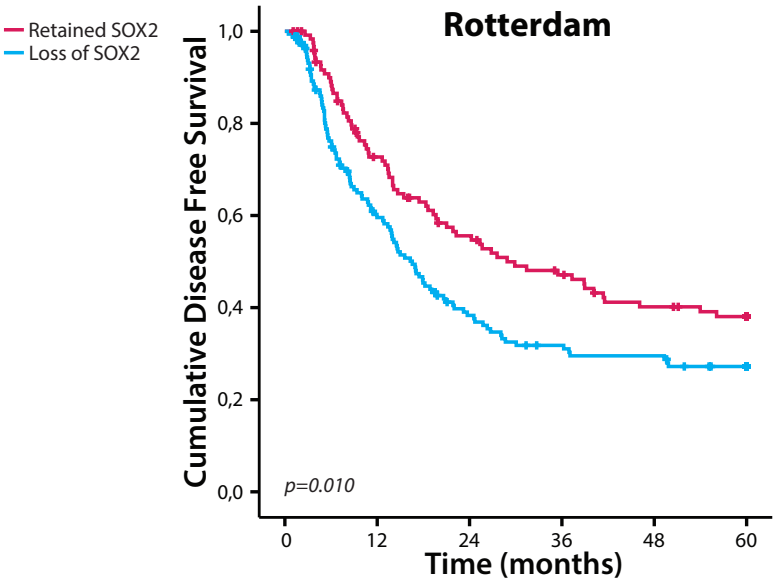
B: Heterogeneous expression of SOX2



C: Presence of SOX2 expression



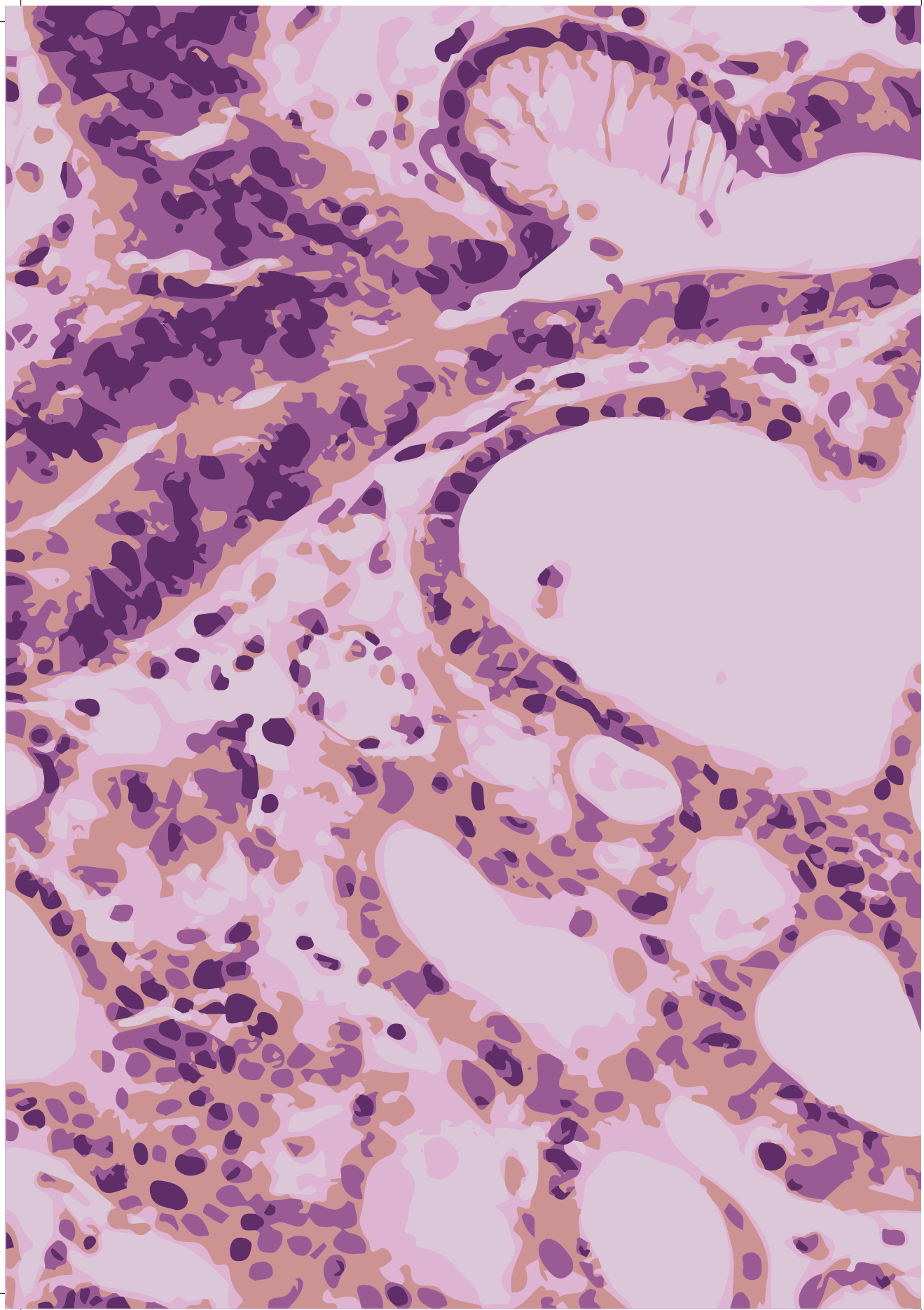
SUPPLEMENTAL FIGURE 2: Representative examples of SOX2 immunohistochemistry, A: SOX2 expression is absent in the tumor cells of esophageal adenocarcinoma, B: an example of heterogeneous expression of SOX2 (>50% of the tumor cells are nuclear positive, therefore interpreted as SOX2 positive), and C: homogeneous presence of nuclear SOX2 positivity.



N at risk

SOX2						
Retained	123	82	60	49	40	36
Loss	165	88	53	42	39	32

SUPPLEMENTAL FIGURE 3: Expression of SOX2 is prognostic for disease free survival in patients with esophageal adenocarcinoma in the Rotterdam cohort, (p-value is indicated in the left lower corner of the graph).



CHAPTER 7

Pattern of P53 protein expression is predictive for survival in chemoradiotherapy-naive esophageal adenocarcinoma

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ABSTRACT

Introduction: *TP53* mutations are considered to be the driving factor in the initiation of esophageal adenocarcinoma (EAC). However, the impact of this gene and its encoded protein as a prognostic marker has not been definitely established yet.

Methods: In total, 204 chemoradiotherapy (CRT)-naive patients with EAC were included for P53 protein expression evaluation by immunohistochemistry (IHC) on the resection specimens, categorized as overexpression, heterogeneous or loss of expression, and correlated with disease free survival (DFS) and overall survival (OS) using multivariable Cox regression analysis. In a subset representing all three IHC subgroups mutational status of selected candidate genes (n=33) and high throughput methylation profiling (n=16) was assessed.

Results: Compared to heterogeneous P53 expression, loss and overexpression were both independently predictive for adverse DFS and OS. *TP53* mutational status significantly correlated with the IHC categories (p=0.035). Most of the EAC with loss- or overexpression harbored *TP53* mutations (18/20, representing nonsense and missense mutations respectively). In contrast, 6/13 EAC with heterogeneous expression were *TP53* wild type, of which two demonstrated *MDM4* or *MDM2* amplification. Combined genomic hypomethylation and high frequency of intra-chromosomal breaks was found in a selection of EAC without P53 overexpression.

Conclusion: P53 expression pattern is prognostic for DFS and OS in this historical cohort of CRT-naive EAC. P53 IHC is an informative readout for *TP53* mutational status in EAC with either loss- or overexpression, but not in case of a heterogeneous P53 pattern. Different EAC pathogenesis might exist, related to P53 and other candidate gene status, DNA hypomethylation and intrachromosomal breaks.

INTRODUCTION

Esophageal adenocarcinoma (EAC), being rare before the second half of the 20th century, is nowadays the predominant histological type of esophageal cancer in Western countries [184,185,214]. Presently the prognostication of patients with EAC is largely based on the TNM-classification supplemented with histological criteria [75]. Although this system has its value in the stratification of patients into prognostic groups [81], the outcome for an individual patient is still difficult to predict. This is demonstrated by the fact that up to 27% of the patients with stage IB develop disease recurrence while up to 24% of the patients with stage IIIA EAC will have no disease recurrence after intentionally curative surgery [81]. Therefore, prognostic biomarkers complementing the TNM classification are urgently needed.

The *TP53* gene (OMIM# 191170), first discovered more than 30 years ago, has a cell- and context dependent biological function. It has been reported that P53 is deregulated in most cancer types. Given its central role in the control of proliferation and senescence, it can be assumed to be the driving force of cancers of various types, including EAC [82,83,215]. Several types of stress can lead to P53 dysregulation. In EAC, mutations in *TP53* are detected early in the pathogenesis, likely linked to severe DNA damage in Barrett's esophagus (BE) due to the reflux of mixed gastric and duodenal juice into the esophagus [216]. Recent genome wide studies proposed that EAC precursor lesions containing *TP53* mutations rapidly develop extensive chromosomal instability with subsequent oncogene activation [24,26,27].

Because of its dominant role in the development of EAC, P53 was also tested as a biomarker in EAC precursor lesions and in advanced EAC. There is growing evidence that P53 overexpression is related to dysplasia and independently predictive for progression in BE [51,53,55,56,106,217]. Overexpression is likely due to *TP53* mutations which stabilize the affected protein. "Absence" of P53 staining was described more recently in dysplastic BE [52]. This loss of expression is likely to be related to truncating mutations or to alternative, including epigenetic, mechanisms. Supporting the significance of the loss of expression, a recent IHC P53 study on a large prospective BE cohort revealed a significantly higher rate of progression to high grade dysplasia or EAC in low grade dysplasia harboring P53 overexpression and even higher in BE with absence of P53 expression [51].

In parallel to the EAC precursors, the results of the earlier investigations also suggested significance of P53 in relation to prognosis in advanced EAC [218-220]. However, strong conclusions cannot be drawn because of several limitations, including heterogeneity related to P53 IHC interpretation and patient selection. This may have influenced the outcome of these studies and as such the true biological effect of P53 in the context of disease progression may remain unidentified.

Therefore, the aim of this study was to examine the prognostic value of P53 in a well-defined group of chemo- and radio-therapy-naive EAC, using a validated IHC approach. To further

investigate the putative mechanism(s) involved, a combinatory investigation of expression pattern, mutational status of *TP53* and a selection of other (relevant) genes, as well as high throughput profiling was performed in a subset of EAC.

METHODS

Patient selection

To evaluate the prognostic value of P53 in patients with EAC, a cohort of patients who underwent surgery with curative intent between 1995 and 2006, without prior (neo-) adjuvant treatment, was selected from the Department of Surgery at the Erasmus University Medical Center (Rotterdam, The Netherlands). All patients had pathologically proven pT2-pT4a adenocarcinoma of the esophagus or at the gastro-esophageal junction. Only patients who were alive one month after surgery were included in the analysis to correct for surgical mortality. Clinical and pathological data were prospectively collected, including anatomical tumor location according to Siewert [205], tumor grade, pathological stage, age at surgery, comorbidities, OS and DFS. Tumor grading and staging was performed according to the TNM system as described by the UICC (Union Internationale Contre le Cancer, 2009, 7th edition) [75]. Resection margin positivity was assessed on tumor cells in the resection margin. To ensure reliable classification, all slides were reviewed by an experienced GI pathologist (FK or KB) for depth of invasion.

The hematoxylin-eosin colored slides from the resection specimens were retrieved from the archive of the Department of Pathology at the Erasmus University Medical Center and a representative slide with EAC was selected. The corresponding FFPE block was retrieved and serial 4µm sections for IHC and mutational analysis were mounted on glass slides.

Immunohistochemical analysis

The first slide of each selected FFPE block was stained for P53, ready to use kit (clone BP53-11, Ventana Medical Systems, Roche, Tuscon, AZ, USA). Staining was performed using an automated slide staining system (BenchMark Ultra, Ventana Medical Systems, Roche, Tuscon, AZ, USA), in which the slides were deparaffinized prior to the staining procedure and heat induced epitope retrieval at 97^0 C for 8 minutes. The primary antibody was incubated for 4 minutes, after which this was visualized using Ultraview (Ventana Medical Systems, Roche, Tuscon, AZ, USA) and counterstained with hematoxylin.

For optimal interpretation, representative tumor samples were evaluated by two experienced gastro-intestinal (GI) pathologists (KB and FK) with specific knowledge on P53, based on earlier published extensive IHC studies on EAC and its precursor lesions [51,202]. A tumor sample with known overexpression of P53 was placed as positive control on each slide. Furthermore, normal tissue surrounding the tumor cells were evaluated for their physiological expression of P53, serving as internal control for the sample under investigation. If the positive control material or internal control was negative the slide was disregarded for analysis. The pattern of P53 IHC was scored on all tumors cells present on the slide, based on the percentage of tumor cells with nuclear positivity on a semi-

quantitative 7-point scale: 0%, 1-20%, 21-40%, 41-60%, 61-80%, 81-90% and 90-100% of the tumor cells. If the scores of the two pathologists were discordant, a third board certified pathologist evaluated the slides (MD), after which the final diagnosis was based on the consensus of two of the three pathologists. All pathologists were blinded for clinical and pathological data.

Mutational analysis and high throughput methylation profiling

In total 34 EAC, among them 10 with no expression of P53, 14 with heterogeneous expression (1-60% of the tumor cells positive) and 10 with overexpression (61-100% positive tumor cells), were selected for the targeted gene sequencing. Tumor area was manually macro-dissected from the successive unstained slides, resulting in at least 30% tumor cells. DNA was extracted using proteinase K and 5% Chelex 100 resin [221]. An Ion AmpliSeq custom-made panel was created for selection of genes [222]. This consisted of primers for the entire *TP53* gene supplemented with hotspots or the entire genes known to be frequently altered in EAC (*ARID1A*, *PIK3CA*, *APC*, *DOCK2*, *ELMO1*, *CDKN2A* and *SMAD4*) [24-27]. Sequencing was performed on the Ion Torrent Personal Genome Machine or IonS5 system (Thermofisher Scientific, Hemel Hempstead, UK) according to the manufacturers protocol. In short, libraries were created using the ION AmpliSeq Library Preparation Kit. Template was prepared using the Ion Onetouch Template Kit and sequencing was performed with the Ion Sequencing Kit as described [221]. One sample was excluded from further analysis because of poor DNA quality and high frequencies of formalin artefacts. All other samples showed comparable and reliable sequence read coverage independent from sample age. The sequence variants with a read frequency of less than 5% (homozygous reference) or more than 95% (homozygous non-reference), with an amplicon coverage of less than 50, or a variant coverage of less than 10 reads were excluded from analysis, to eliminate formalin artefacts. All variants found in an intronic, intergenic, non-coding RNA or UTR3/5 region, and synonymous single nucleotide variations (SNV) were excluded.

Sixteen EAC, among them five tumors with loss of expression, five with overexpression and six with heterogeneous P53 expression, were selected for genome-wide methylation analysis in addition to the targeted sequencing. Therefore, the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA, USA), targeting over 850,000 methylation sites, was applied according to the manufacturer's instruction at the Microarray unit of the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ, Heidelberg, Germany). For a detailed description see earlier publication [223]. For unsupervised clustering the most differential probes (with 0,22 SD difference from the mean) were selected. To assess copy number variation (CNV) methylation data were implemented in the R/Bioconducter packages Conumee. Intra-chromosomal breaks were calculated from the number of segments defined by the Conumee package (blue horizontal lines in supplementary figure

3). Segments are defined as chromosomal regions with distinct copy number changes to the adjacent region. The number of segments relative to the median number of segments within this sample series was determined for each sample (presented in Figure 4). With this method amplification of genes were also assessed as described earlier.[224] To validate amplification of *MDM2* immunohistochemistry staining (clone 1F2, Merck Milipore, Amsterdam, Holland) was performed on all samples in which no *TP53* mutation was found.

Ethics

The investigational protocol was approved by the medical ethical committee in the Erasmus Medical Center (Rotterdam, The Netherlands) (MEC-12-469).

Statistical analysis

The primary endpoint of the study was 5-year DFS, defined as the time between surgery and the first clinical recurrence of disease, defined as clinical or radiological evidence of disease recurrence. Patients lost to follow-up were censored at the time of the last visit to the outpatient clinics. Secondary endpoint was OS, defined as time between surgery and death. The optimal cut-off for IHC was calculated using a ROC-curve and corresponding Youden-index (Supplemental Figure 1 and Supplemental Table 2).

The interobserver variation for the assessment of P53 staining between the two pathologists was calculated using Cohen's kappa. Strength of agreement was categorized as follows: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, good; and 0.81–1.00, excellent.

Kaplan Meier curves were used to plot the 5-year DFS by P53 status. Uni- and multivariable Cox proportional hazard models were applied to calculate the association between P53 IHC and survival. In the multivariable analysis adjustments were made for all clinical and pathological factors which proved to be prognostic for survival in the univariable analysis ($p<0,05$). The pN-stage was dichotomized in pN0 and a pN+ (pN1-3) group for the Cox regression analysis. The P53 status and mutational status were correlated using Fisher's Exact test. The analysis was performed using SPSS-software (version 22, SPSS IBM inc, Armonk, NY, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics

Two hundred and sixteen (216) patients were initially identified to be eligible for this study. Of 12 patients, the formalin-fixed paraffin-embedded (FFPE) blocks could not be retrieved and were therefore excluded. From the remaining 204 patients with EAC the majority had a pT3-tumor (85.3%), tumor positive lymph nodes (79.4%) and negative resection margins (62.7%). Detailed patient and tumor characteristics are shown in Table 1 and Supplemental Table 1.

P53 expression correlates with Overall - and Disease Free Survival

The optimal cut-off for P53 expression was calculated, based on the receiver operating characteristics (ROC) curve and Youden-index (see Supplemental Table 2 and Supplemental Figure 1), into three groups, namely loss of expression (0% of tumor cells positive), heterogeneous expression (1-60% of tumor cells positive) and overexpression (61-100% of tumor cells positive). The interobserver variation for the assessment of P53 between the two observers was excellent (kappa 0.850, p<0.001). From the 204 patients, 55.9% (n=114) of the EAC showed overexpression, 26.5% (n=54) loss of expression, while 17.6% (n=36) had a heterogeneous expression. In all cases this was a homogeneous expression pattern throughout the cancer, of which representative examples are shown in Figure 1.

The pattern of P53 expression associated with disease free survival (DFS); overexpression - median DFS 14.6 months (95% CI 10.0-19.2), loss of expression - median DFS 14.2 months (95% CI 7.9-20.5) compared to the group with heterogeneous P53 expression - median DFS 37.1 months (95% CI 24.3-49.9). The corresponding Kaplan-Meier curves are shown in Figure 2.

Univariable analysis demonstrated a correlation between P53 expression and DFS (p=0.036). The risk of recurrence of EAC was increased for patient with P53 overexpression (hazard ratio (HR) 1.91; 95% CI 1.16-3.14) as well as loss of P53 expression (HR 1.57; 95% CI 0.9-2.74) compared to heterogeneous P53 expression. This was also significant after multivariable analysis, adjusted for pT-stage, pN-stage, tumor differentiation and resection margin status (p=0.001). Patients with P53 overexpression/loss showed a significantly worse DFS compared to heterogeneous expression (HR 2.61; 95% CI 1.57-4.32; p= <0.001 and HR 2.75; 95% CI 1.55-4.9; p= <0.001, respectively) (Table 2 and Supplemental Table 3). A shorter overall survival (OS) was associated with P53 overexpression (median OS 19.4 months (95% CI 14.3-24.5)), and loss of expression (median OS 18.5 months (95% CI 15.3-21.7)) compared to the group with heterogeneous expression (median OS 32.4 months (95% CI 23.0-41.8)). Although no significance was identified in the univariable analysis (p=0.265),

TABLE 1: Clinico-pathological characteristics for the 204 included patients with esophageal adenocarcinoma.

	All patients n=204		P53 Loss (0%) n=54		P53 Heterogeneous (1-60%) n=36		P53 Overexpression (61-100%) n=114		
	N	%	N	%	N	%	N	%	p-value
Age at surgery									
Median	64.0		63.0		68.5		64.0		0.462
Range (IQR)	55.3-72.0		55.0-72.0		56.3-74.0		55.0-72.0		
Sex									
Male	174	85.3	51	25.0	29	14.2	95	46.6	0.337
Female	30	14.7	5	2.5	7	3.4	17	8.3	
Siewert classification									
Type 1	75	36.8	23	11.3	11	5.4	41	21.1	0.576
Type 2	129	63.2	33	16.2	25	12.3	71	34.8	
Pathologic T-stage									
pT2	27	13.2	9	4.4	3	1.5	16	7.8	0.556
pT3 or pT4	177	86.8	47	23.0	33	16.2	96	47.1	
Pathologic N-stage									
pN0	42	20.6	16	7.8	5	2.5	22	10.8	0.207
pN1 or more	162	79.4	40	19.6	31	15.2	90	44.1	
Histology grade									
Well	5	2.5	3	1.5	1	0.5	1	0.5	0.498
Moderate	80	39.2	22	10.8	15	7.4	43	21.1	
Poor	119	58.3	31	15.2	20	9.8	68	33.3	
Resection margin status									
pR0	128	62.7	33	16.2	22	10.8	73	35.8	0.714
pR1	76	37.3	23	11.3	14	6.9	39	19.1	
Alive after 60 months									
Yes	34	16.7	10	4.9	8	3.9	16	7.8	0.518
No	170	83.3	46	22.5	28	13.7	96	47.1	

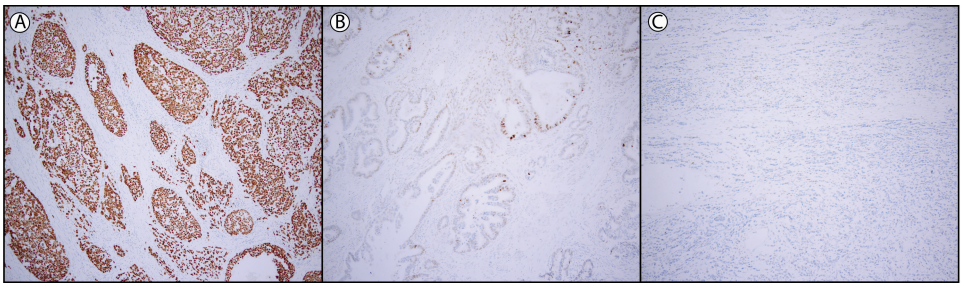


FIGURE 1: Examples of P53 expression in esophageal adenocarcinoma. A: overexpression (61-100% positive tumor cells) B: heterogeneous expression (1-60% positive tumor cells) and C: loss of expression (0% positive tumor cells). Magnification 1:100.

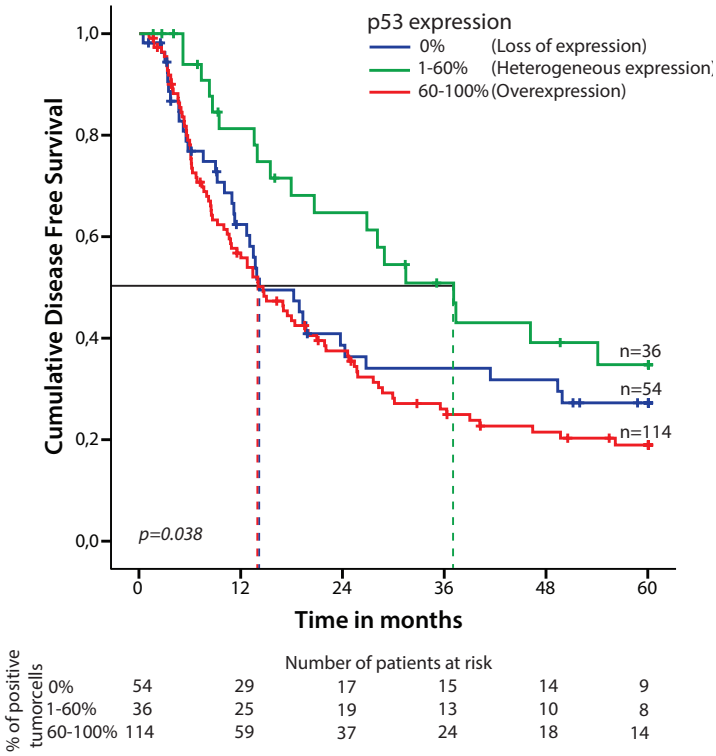


FIGURE 2: Kaplan-Meier curve for Disease Free Survival in chemoradiotherapy-naive patients with esophageal adenocarcinoma. Expression pattern of P53 is subdivided into three groups: 0% of the tumor cells positive (loss of expression), 1-60% of the tumor cells positive (heterogeneous expression) and 61-100% of the tumor cells positive (overexpression). The dotted line indicates the median survival for each of the three groups. Number of patients at risk is indicated for each of the three groups at the bottom of the figure.

the multivariable analysis demonstrated that P53 expression was significantly associated with OS (p=0.003). Overexpression and loss of P53 expression were prognostic for a shorter survival period (HR respectively 1.99; 95% CI 1.29-3.07; p=0.002 and 2.17; 95% CI 1.33-3.55; p=0.002) compared to heterogeneous expression (Table 2 and Supplemental Table 3).

TABLE 2: Multivariable Cox regression analysis for Disease Free Survival and Overall Survival in patients with esophageal adenocarcinoma

	Multivariable Cox regression analysis					
	Disease Free Survival			Overall survival		
	HR	95% CI	p-value	HR	95% CI	p-value
Age	NA	NA	NA	1.026	1.010-1.042	0.001
pT-stage (ref pT2)						
pT3/4	2.152	1.156-4.005	0.016	2.010	1.168-3.459	0.012
pN-stage (ref pN0)						
pN+	3.445	1.981-5.990	<0.001	2.434	1.560-3.796	<0.001
Differentiation (ref good to moderate)						
Poor	1.467	1.016-2.119	0.041	1.551	1.112-2.165	0.010
Resection margin (ref pR0)						
pR+	1721	1.192-2.484	0.004	1.716	1.230-2.393	0.001
P53 (ref heterogeneous)						
Loss of expression	2.754	1.547-4.903	0.001*	2.174	1.333-3.546	0.003*
Overexpression	2.605	1.571-4.320		1.989	1.288-3.071	

HR=Hazard Ratio, CI=Confidence Interval, NA = not available, excluding patients who died within one month after surgery. P53 expression, based on immunohistochemical expression, was classified as loss of expression (0% of the tumor cells positive), heterogeneous expression (1-60% of the tumor cells positive) and overexpression (61-100% of the tumor cells positive). *global p-value

Targeted mutational analyses and high throughput methylation profiling

To shed light on the possible mechanism(s) underlying the P53 staining patterns, sequencing of the whole *TP53* gene was performed using the Ion Torrent platform on 33 selected EAC (10 with overexpression, 10 with loss, and 13 with a heterogeneous expression) (Supplemental Table 4). Overall, 25 of 33 (76%) EAC showed a *TP53* mutation. *TP53* status correlated significantly with the IHC staining pattern (p=0.035) (Figure 3 and Supplemental Table 5). Of the 10 cases with loss of expression eight had non-sense mutations (splice site, frameshift mutation or stopgain) and two no mutation. All EAC with overexpression of P53 as detected by IHC had missense mutations. The EAC within the heterogeneous P53 expression group demonstrated a mixed picture, representing the three different patterns. Those with more than 40% P53 positive tumor cells all showed missense mutations (n=3), in analogy to EAC with overexpression, while in the lower percentage category two out

of four showed a nonsense mutation (one containing both a splice site and stopgain mutation). EAC cases with heterogeneous P53 expression in the middle group (n=6, 21-40%) demonstrated no underlying *TP53* mutations in four and two nonsense mutations. Besides *TP53*, in total, 21 other proven pathogenic mutations in the following genes *SMAD4* (n=7), *ARID1A* (n=5) , *PIK3CA* (n=2), *DOCK2* (n=6) and *ELMO* (n=1) were detected, significantly more in EAC with a heterogeneous P53 expression (13/21; p=0.032) (Figure 3 and Supplemental Table 5). In these samples no mutations in CDKN2A were detected. Four cases of our series revealed no mutation in the investigated genes (cases 21, 22, 28 and 29). Multiple mutations were identified (including *TP53*) in 15 EAC, predominantly again in the heterogeneous P53 expression group (9/13 versus 3/10 and 3/10, respectively). In addition, a subset of these EAC (n=16) were investigated using high throughput methylation profiling for the detection of chromosomal alterations between the three groups [26], including five with overexpression, five with loss and six with a heterogeneous expression, all with known *TP53* mutational status (see Figure 4). No hypermethylation of the promotor region of *TP53* was detected in any of these EAC, including the two cases with loss of P53 expression and wild type (not mutated) *TP53* (cases 18 and 29). Based on copy number variations (CNV) derived from these high throughput methylation profiles (see Material and Methods section), regional chromosomal amplifications were identified, including those encompassing for example *MDM2* and *MDM4*, two genes of which amplification is known to be related to an alternative inactivation of P53 besides mutations. Two EAC showed such an amplification (cases 21 and 22, for *MDM2*, confirmed by immunohistochemistry, and *MDM4*, respectively, see supplementary Figure 2). No other mutations were identified in these cases, and both showed a heterogeneous P53 expression (21-40% of positive tumor cells) (Figure 3 and 4). Besides these specific amplifications, an unsupervised clustering of the top 10,454 most differentiating CpG-sites was performed (see Figure 4 (heatmap) and Supplementary Figure 3 (Violin plots)). No difference was identified for the overall methylation distribution between the EAC investigated (Supplementary Figure 3, bottom panel), while a clear hypomethylation profile was identified for the most differentiation CpG-sites in seven EAC out of the 16 cases. These included three with absence, three with a heterogeneous and one with overexpression of P53. Only one showed no *TP53* anomaly (case 18, no P53 expression), while all others demonstrated either a mutation in *TP53* itself (three nonsense, one missense), or amplification of *MDM2* or *MDM4*. In addition, the number of intrachromosomal breaks per individual EAC was scored based on the CNV profile (see Supplementary 2 and Figure 4). This analysis demonstrated that six out of the seven EAC with a hypomethylation profile showed a higher number of breaks compared to the group median, i.e., indicated in red boxes in Figure 4 (including those with the *MDM2* and *MDM4* amplification), while this was observed for only two of the EAC within the non-hypomethylated group. These data suggest that there is a correlation between P53 status

(protein expression, mutational profile and *MDM2/4* amplification), accumulation of other mutations (preferentially in the P53 heterogeneous staining group), preferential presence of a hypomethylated profile in the loss and heterogeneous P53 group, and occurrence of intrachromosomal breaks.

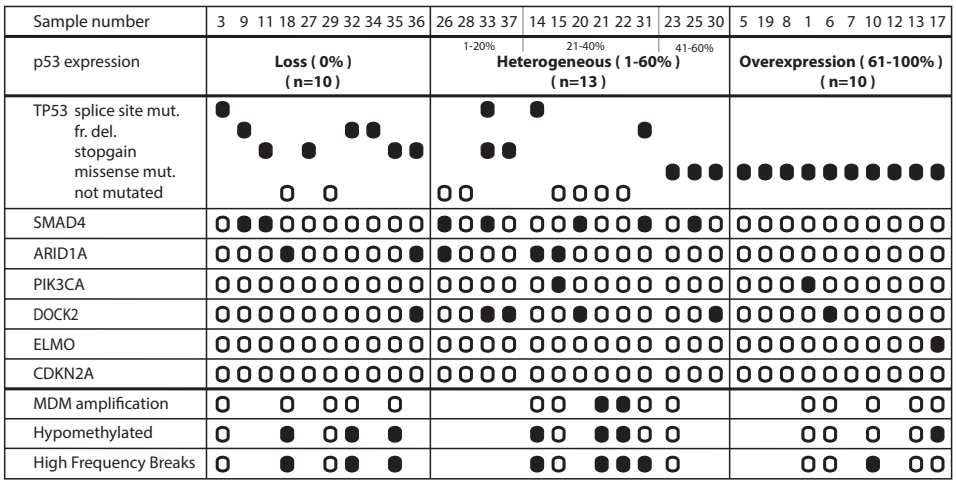
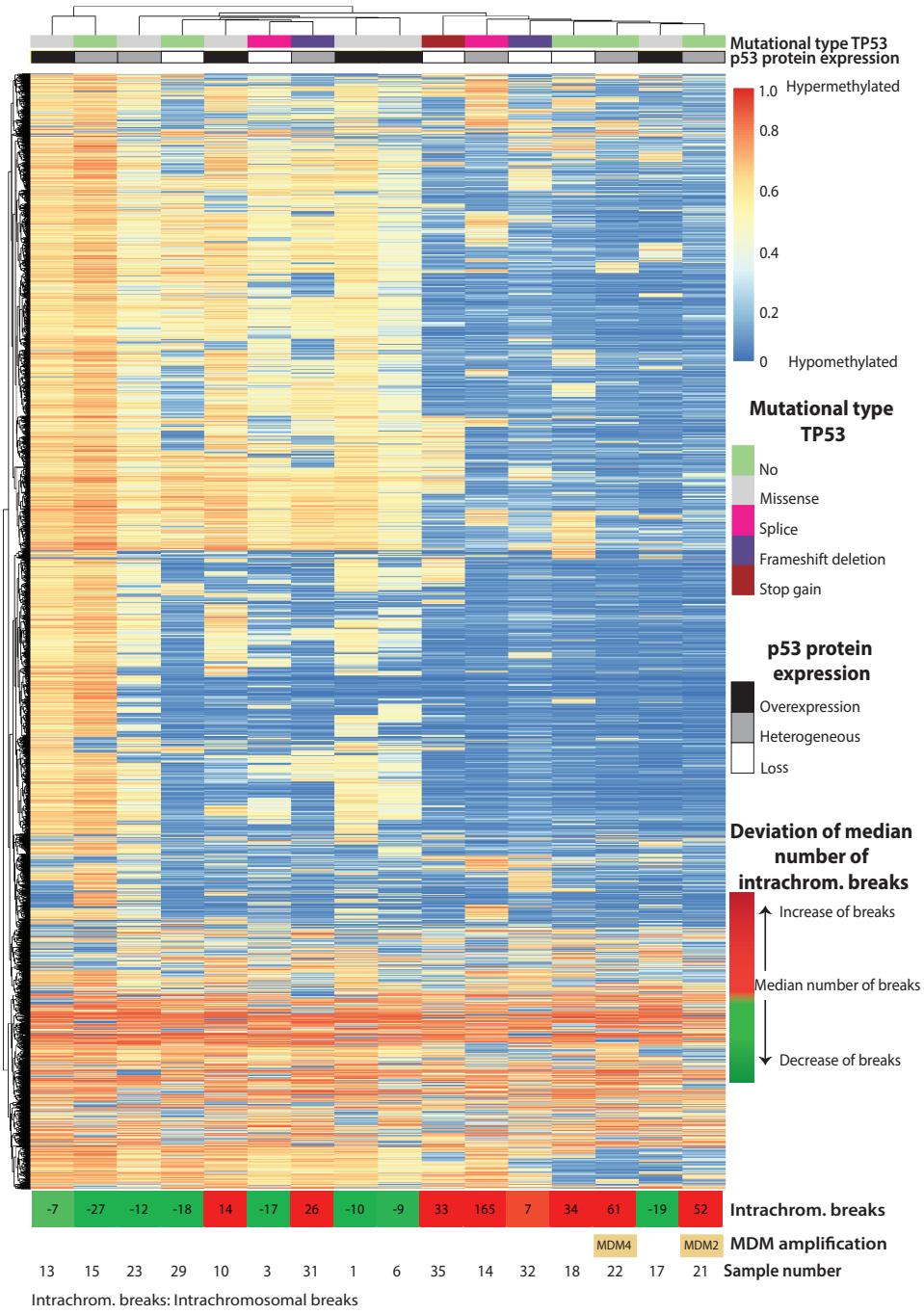


FIGURE 3: Molecular profile of esophageal adenocarcinoma. Mutational profile, as detected by targeted sequencing in 33 cases is categorized by P53 expression pattern. The order of samples is determined by the percentage of positive P53 tumor cells. The exact mutations found are displayed in supplementary Table 4, ordered by case number. The CpG methylation-derived information (copy numbers, hypo-methylation status and relative high frequency breaks are summarized here. Further details are provided in the supplementary figures 2 and 3.

FIGURE 4: Unsupervised clustering of CpG methylation of 16 esophageal adenocarcinomas, using top 10,454 differential probes, in which every row is a methylation probe and every column is a sample. The corresponding violin plots for all methylation probes as well as top 10,454 differential probes are depicted in Supplementary Figure 3. At the top of the image, P53 expression and TP53 mutational status are indicated for each sample. In the bottom of the image, the deviation of the median number of intra-chromosomal breaks (median number of intra-chromosomal breaks are calculated, samples with less breaks are depicted in green, and samples with more breaks depicted in red, numbers indicate deviation from median number of intra-chromosomal breaks) as well as the two samples with MDM2 and MDM4-amplification are indicated. Five samples with an altered P53 pathway (either TP53 mutations or MDM2/4 amplifications) show extensive intra-chromosomal breaks (sample 31, 35, 14, 22 and 21), and only one sample without alteration of the P53 pathway (sample 18) showed extensive intra-chromosomal breaks. However none of the six samples (sample number 13, 23, 10, 1, 6 and 17) with a missense mutation showed an increase of intra-chromosomal breaks. Increased number of intra-chromosomal breaks correlates to the clustered hypomethylated CpG-sites.



Intrachrom. breaks: Intrachromosomal breaks

DISCUSSION

This study primarily aimed to evaluate the relevance of P53 IHC for survival of patients with advanced EAC. A large, well defined cohort of CRT-naive surgically treated EAC was analyzed, and the pattern of P53 expression was shown to be significantly correlated with DFS and OS, independently from other clinic-pathological parameters including tumor stage. In addition, P53 expression patterns were correlated with the underlying *TP53* mutational status and genome wide methylation profile and derived information on chromosomal anomalies.

TP53 is one of the driving genes for the progression of BE into adenocarcinoma and whole genome sequencing studies have detected a high mutation frequency of *TP53* in EAC [25,27,29]. Conflicting results have, however, been reported so far on *TP53* and survival in patients with EAC [54,219,220,225-229]. Three previous systematic reviews analyzed the current literature and performed a meta-analysis of up to 16 different studies, employing IHC or sequencing of the *TP53* gene [82,83,215]. Although, overall, similar results were reported in all three meta-analyses suggesting a negative effect of mutated *TP53* on prognosis, the data should be interpreted with caution. First of all, many of the earlier studies did not consider the bias of patient selection and chemoradiotherapy (CRT) treatment [218,228,230-232]. Several studies included patients who received surgery only as well as patients who underwent neoadjuvant treatment or definite CRT. This is of importance since P53 might modulate CRT response as suggested in earlier studies [218,230-237]. Another important limitation of the published studies is the inconsistent methodology for detection and classification of P53 expression. From five studies using IHC on homogeneous EAC cohorts (total 384 patients), with surgery as single treatment modality and IHC approach, none qualified loss of expression as aberrant [219,220,226,227,229] (see Supplemental Table 6). This is significant since according to our interpretation, around 26% of EAC showed loss of P53 expression and had significantly worse outcome.

In the present study based on evaluation of EAC resection specimens of 204 CRT-naive patients, with surgery as single modality, P53 was detected by IHC and categorized by experienced observers using optimized cut-off values. The pattern was classified as heterogeneous, overexpression or loss of expression.

Until now it is not clear whether P53 IHC or sequencing of *TP53* is the most optimal tool to improve risk stratification in EAC. Mutational status was suggested to be preferable by a recent meta-analysis [215]. Several previous EAC studies applied mutational status as single read out [218,220,230]. The assays used for gene sequencing in those older studies are likely to be suboptimal, since the *TP53* gene was only partly sequenced using PCR-based methods, which correlates with the low mutational rate (40-50%) [218,220,230]. Although the efficacy of the gene sequencing techniques improved in recent years, they are still more time-consuming, labor intensive and expensive compared to IHC. Prediction of mutational status by IHC

could be an alternative, but the prognostic accuracy might depend on the underlying cancer type [238]. To study the correlation between protein expression pattern and genetic status, a subset of 33 EAC was investigated using a targeted next generation sequencing approach. *TP53* mutational frequency rate was 76%, which is comparable to the recent investigations using whole genome or exome sequencing techniques [25,29]. *TP53* status significantly correlated with the defined IHC categories (p=0.035). EAC with heterogeneous P53 expression was also heterogeneous in terms of the underlying *TP53* status, although it seems to be (again) subdivided into three groups, similar to loss of expression, similar to overexpression, and the (remaining) intermediate group. Of interest is that most additional mutations in the other candidate genes investigated were identified in the group with heterogeneous P53 expression, including two cases with regional amplifications of *MDM2* or *MDM4* (Figure 3). These were identified in EAC without any other mutation. In contrast, all EAC with high percentage of P53 positive cells (more than 61%, n=10) showed missense mutations in *TP53*, which is in line with results of two earlier studies [220,239]. EAC with loss of P53 expression demonstrated predominantly nonsense mutations, including splicing, stopgain and frameshift mutations (8/10). These nonsense mutations were also observed in a subset of EAC with a heterogeneous, but relatively low to modest P53 expression, in fact three out of five cases. In 4 out of five of the remaining cases no *TP53* mutation was found. These observations warrant additional studies to be performed.

The putative difference in pathogenesis between these subgroups is supported by the results of the high throughput methylation profiling performed. The hypomethylated profile of the most differentiating CpG sites combined with a high frequency of intrachromosomal breaks was predominantly observed in EAC with loss or a heterogeneous P53 pattern (either by a nonsense mutation (n=3) or *MDM2/4* amplification (n=2)). No apparent differences were observed using all CpG targets, demonstrating its specificity. EAC with a hypomethylated profile showed a higher frequency of intrachromosomal breaks, indicative for chromosomal instability. This is in line with the recently suggested role of DNA methylation as the newly identified guardian of the genome [240]. Based on this small subset of patients, these observations might be a potential explanation for the differences in DFS and OS as found in the present study, which warrants further investigations. Besides the prognostic effect of P53 expression, our results are clinically important. *TP53* status might be predictive for response to neoadjuvant chemotherapy [202,218]. Clinical trials, such as the PANCHO trial, stratified for *TP53* status, are underway and have completed recruitment [76]. Other studies rely on new therapeutic agents created to restore the wild type activity of P53, one of the most promising compounds being APR-246 [241]. Here we show that if IHC is used as a read-out for mutational status, results should be interpreted with caution especially in

EAC with a heterogeneous P53 expression. In contrast, EAC with P53 overexpression or loss of expression are likely to have an underlying somatic mutation and extensive sequencing might not be necessary.

There are some limitations to this study. *TP53* sequencing was done in a single EAC area, and therefore potential intratumoral heterogeneity was not accounted for. However, this is considered unlikely to play an important role, since identical *TP53* mutations and homogeneous loss of heterozygosity of the *TP53* locus were detected across separated tumor regions in EAC previously [222], and a homogenous IHC was identified in all cases. Furthermore, although P53 is stained using a proven informative automatic staining system and a standardized protocol, the scoring is subjective in nature. However, the interobserver variation for P53 IHC was excellent.

In summary, this study leads to various conclusions. First of all, we have demonstrated that P53 expression pattern is significantly correlated with DFS and OS. This finding stresses the biological role of P53 for the prognosis of patients with EAC. Secondly, we have shown that IHC is a good read out for the presence of *TP53* mutations mainly in EAC with P53 overexpression and probably in EAC with loss of expression but not in EAC with a heterogeneous P53 expression. This might be important for current and future studies in which patient treatment is stratified according to the *TP53*/ P53 status. In addition, our study could suggests existence of different pathogenesis of EAC, related to the P53 pathway (*TP53* mutational status and *MDM2/4* amplification), with downstream additional mutations of other candidate genes, as well as DNA methylation alterations and possibly related chromosomal instability. Yet, more work needs to be done for accurate genetic classification of EAC to fully reveal prognostic genetic signatures and involved mechanisms.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL TABLE 1: Basic clinico-pathological characteristics for all patients subjected to mutational analysis.

	TP53 sequencing (n=33)	
	N	%
Age at surgery		
Median	63.00	
Range (IQR)	54.50-71.50	
Sex		
Male	31	93.9
Female	2	6.1
Siewert classification		
Type 1	12	36.4
Type 2	21	63.6
Pathologic T-stage		
pT2	3	9.1
pT3 or pT4	30	90.9
Pathologic N-stage		
pN0	6	18.2
pN1 or more	27	81.8
Histology grade		
Well	1	3.0
Moderate	10	30.3
Poor	22	66.7
Resection margin status		
pR0	22	66.7
pR1	11	33.3
Follow-up time, months		
Median	19.8	
Range (IQR)	9.63-41.33	
P53 expression		
0%	10	30.3
1-60%	13	39.4
61-100%	10	30.3

SUPPLEMENTAL TABLE 2: Calculation of optimal cut-off for % nuclear positive tumor cells for P53 immunohistochemistry.

% of P53 positive tumor cells	sensitivity	specificity	Youden-index
1-20	1	0	0
21-40	0,944	0,127	0,071
41-60	0,894	0,206	0,1
61-80	0,866	0,27	0,136
81-90	0,775	0,317	0,092
91-100	0,69	0,397	0,087
0	0,254	0,698	-0,048

SUPPLEMENTAL TABLE 3: Univariate Cox regression analysis for Disease Free Survival and Overall Survival in neoadjuvant treatment naïve patients with esophageal adenocarcinoma.

	Univariable Cox regression analysis					
	Disease Free Survival			Overall survival		
	HR	95% CI	p-value	HR	95% CI	p-value
Age	1.007	0.990-1.024	0.412	1.017	1.002-1.033	0.026
Sex (ref male)						
Female	1.172	0.750-1.833	0.486	0.942	0.612-1.451	0.786
Weight	0.990	0.977-1.003	0.142	0.992	0.981-1.004	0.171
Siewert (ref Type I)						
Type II	0.859	0.612-1.205	0.379	0.871	0.639-1.186	0.380
pT-stage (ref pT2)						
pT3/4	2.723	1.503-4.932	0.001	2.394	1.427-4.014	0.001
pN-stage (ref pN0)						
pN+	3.504	2.044-6.007	<0.001	2.460	1.602-3.778	<0.001
Differentiation (ref well to moderate)						
Poorly	1.716	1.216-2.421	0.002	1.544	1.134-2.104	0.006
Resection margin (ref pR0)						
pR+	2.143	1.528-3.005	<0.001	2.101	1.540-2.867	<0.001
P53 (ref heterogeneous)						
Loss of expression	1.569	0.897-2.743	0.036	1.333	0.831-2.138	0.265
Overexpression	1.909	1.161-3.139		1.420	0.931-2.165	

HR=Hazard Ratio, CI=Confidence Interval, patients who died within one month of surgery were excluded, P53 immunohistochemistry assessed as loss (0% of the tumor cells positive), heterogeneous expression (1-60% of the tumor cells positive) and overexpression (61-100% of the tumor cells positive)

SUPPLEMENTAL TABLE 4: Summary of mutations found by Ion Torrent Sequencing of our custom made gene panel. See: http://www.oncotarget.com/index.php?journal=oncotarget&page=article&op=downloadSuppFile&path%5B%5D=22021&path%5B%5D=28469.

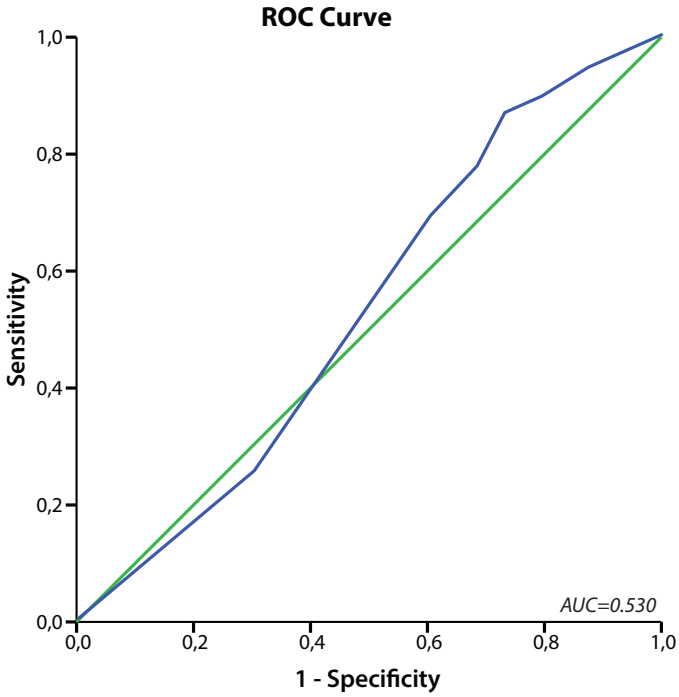
SUPPLEMENTAL TABLE 5: Number of TP53 mutations and other mutations (SMAD4, ARID1A, PIK3CA, DOCK2, ELMO and CDKN2A) found by Next Generation Sequencing specified by tumors with aberrant (combined loss of expression and overexpression) and heterogeneous expression of P53 immunohistochemistry (IHC). Difference is calculated by Fisher exact test.

	P53 IHC expression		
	Loss and Overexpression	Heterogeneous	p-value
TP53 mutated	18	7	0.035
TP53 not mutated	2	6	
Other mutations	7	10	0.032
No other mutations	13	3	

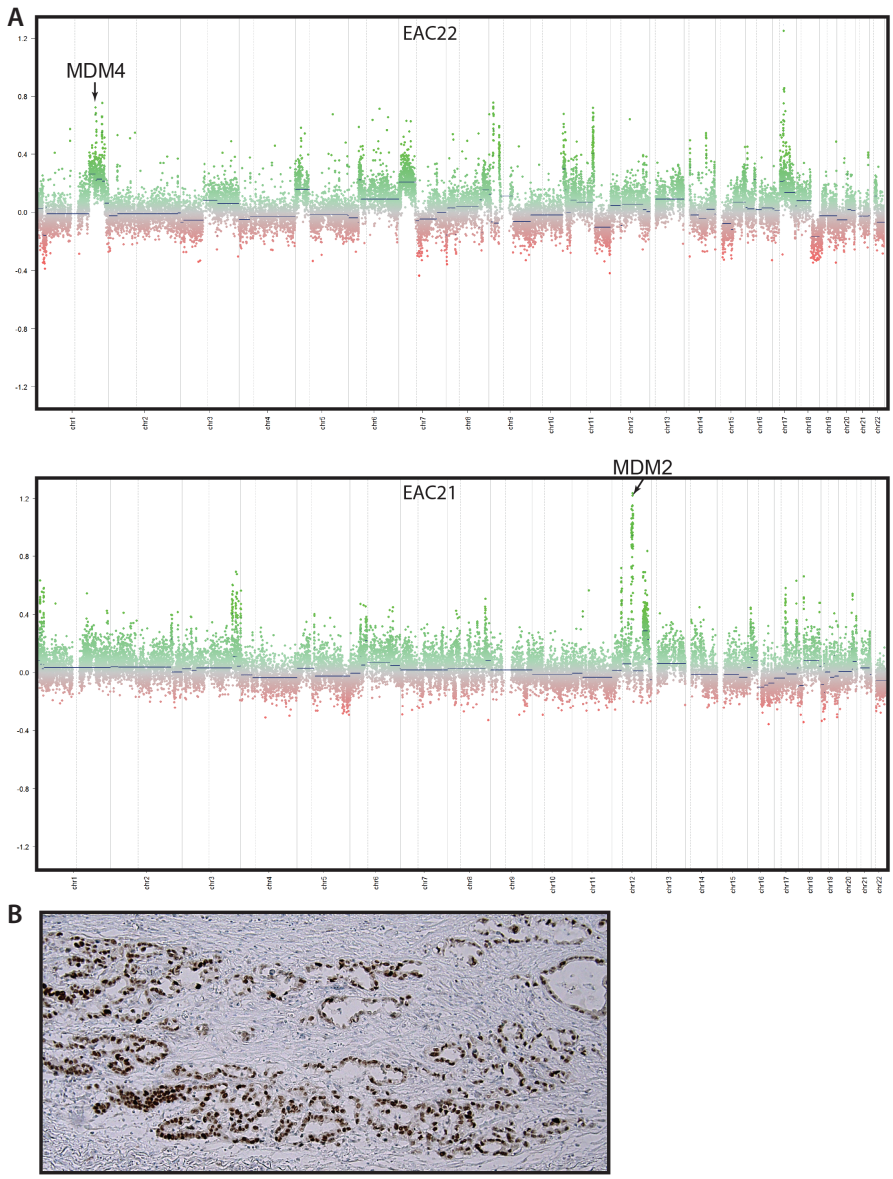
SUPPLEMENTAL TABLE 6: Characteristics of various earlier studies on the prognostic value of P53 in patients with esophageal adenocarcinoma (EAC).

First Author [Reference]	Year	n EAC (% of total included patients)	Specimen	CRT	P53 clone antibody used	Cut-off for overexpression	Loss of expression considered?	Predictive
Flejou[229]	1994	62 (100%)	Resection	No	DO7	NA	No	No
Aloia[225]	2001	44 (72%)	Resection	No	PAb1801	NA	No	Yes
Falkenback[227]	2008	59 (100%)	Resection	No	DO7	5%	No	No
Madani[220]	2010	142 (100%)	Resection	No	DO7	1%	No	Yes
Cavazzola[226]	2009	46 (100%)	Resection	No	DO7	10%	No	No
Lehrbach[219]	2009	75 (100%)	Resection	No	DO7	<2 on scale of 5	No	No
Fareed[228]	2010	245 (94%)	TMA	Yes	NA	10%	No	Yes*
Duhaylongsod[242]	1995	42 (100%)	Resection	Yes	PAb1801	NA	No	No
Sauter[236]	1995	24 (100%)	Biopsy +resection	Yes	PAb1801	>5 adjacent cells in 1HPF	No	Yes
Moskaluk[54] §	1996	88 (100%)	Resection	Yes	DO7	50%	No	No
Wu[232] §	1998	92 (100%)	Resection	Yes	DO7	50%	No	No
Ribeiro[239]	1998	42 (74%	Resection	Yes	DO7	Weak positive	No	No

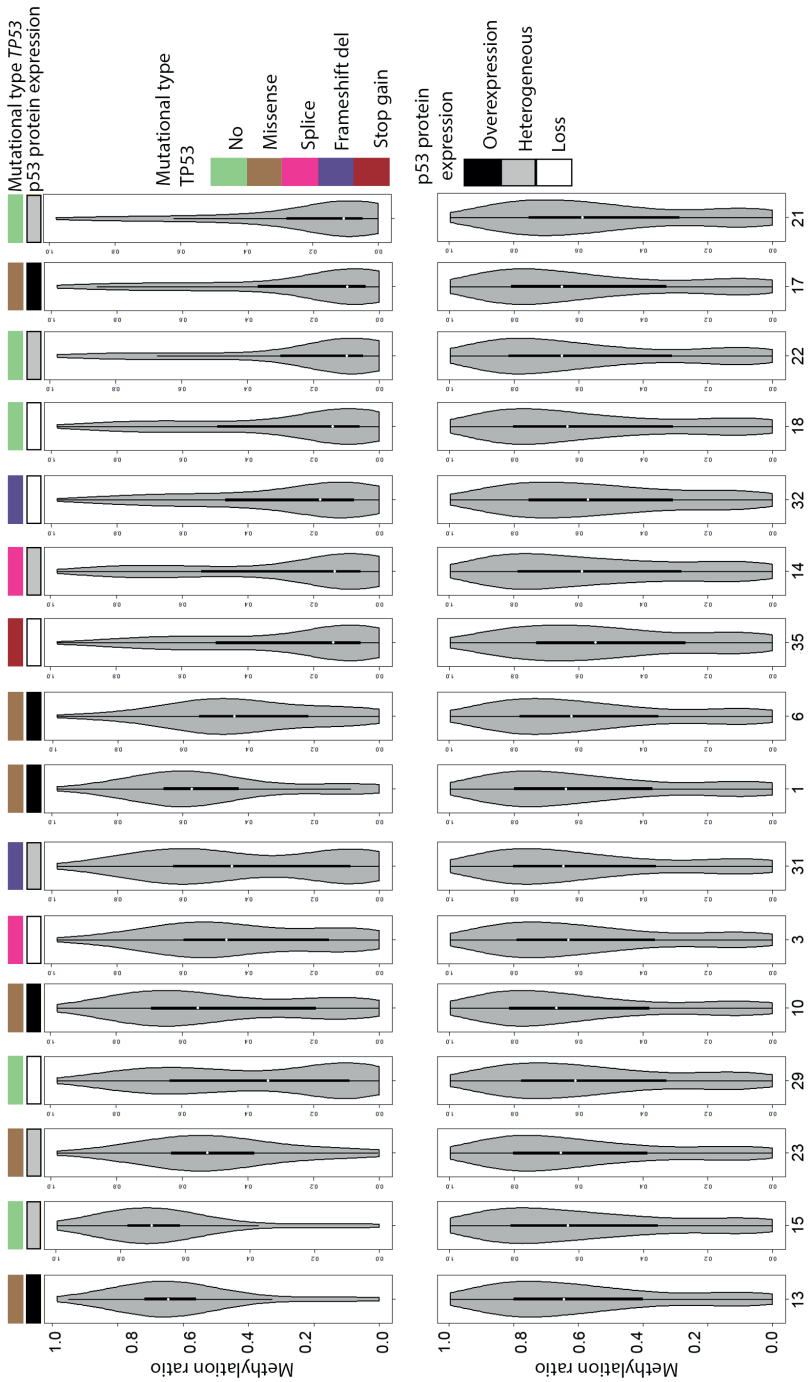
*: in patients treated with chemotherapy P53 was not predictive for survival. §: Overlap in patient group. NA: Not Available. CRT: chemoradiotherapy.



SUPPLEMENTAL FIGURE 1: Receiver Operating Characteristics-curve for the semi-quantitative P53 expression, according for the % of nuclear positive tumors cells, which is used to calculate the optimal cut-off value of P53 expression.



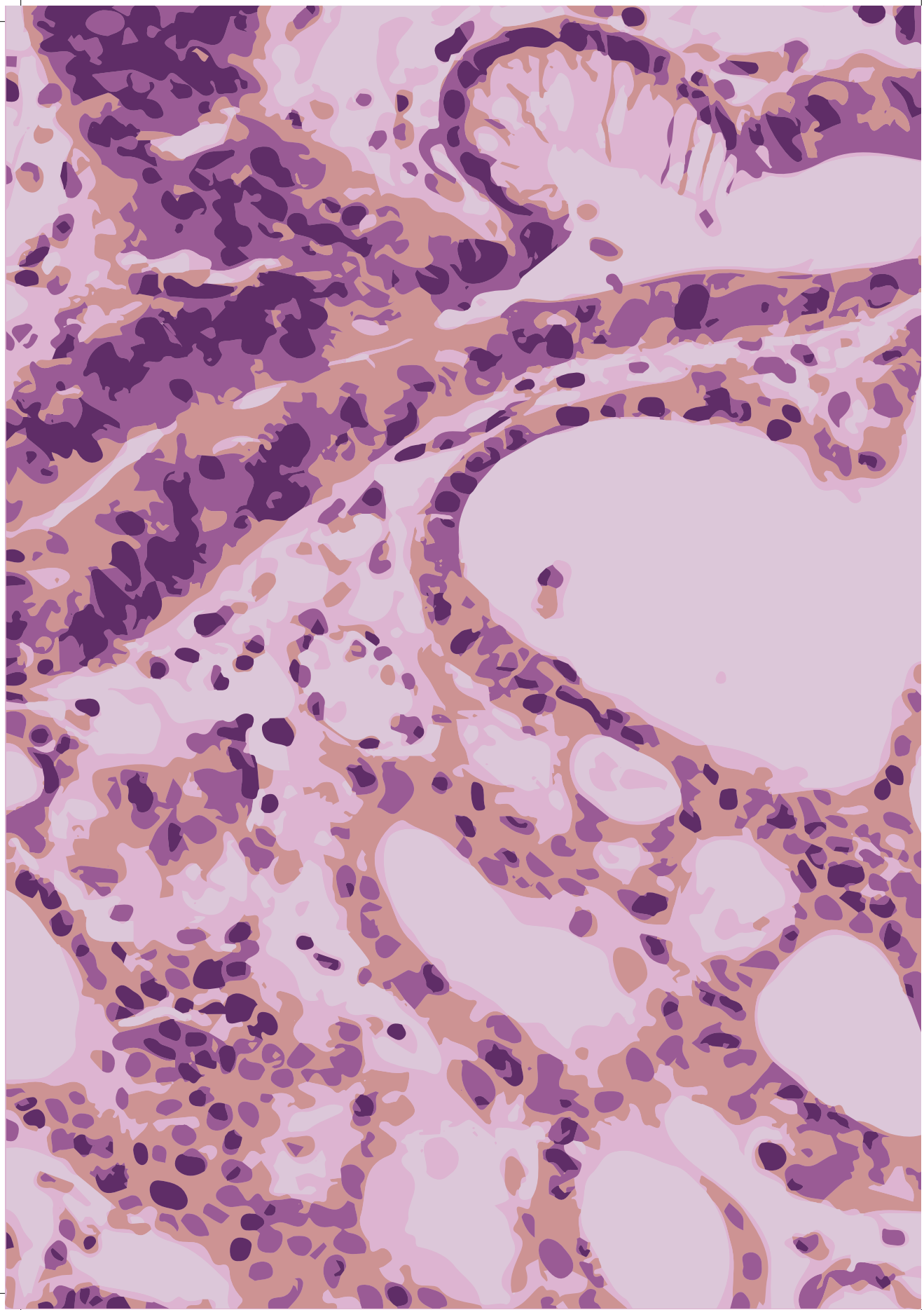
SUPPLEMENTAL FIGURE 2: A: Copy number profiles of selected esophageal adenocarcinoma (EAC) cases (sample number 21 and 22). Methylation intensity data were used to calculate relative copy numbers (output of the Conumee software package). Under- (red) and over-represented (green) regions are highlighted. The positions of the MDM2 and MDM4 amplicon peaks are indicated. The blue lines represent the regions within chromosomes (segments) with similar copy number. The total number of segments was determined per sample to estimate the relative frequency of intrachromosomal breaks in each case. B: MDM2 amplification of sample number 21 was validated by immunohistochemical staining, magnification 100x.



SUPPLEMENTARY FIGURE 3: Violin plots of the selected and overall CpG methylation data. Upper panel: the methylation profile of the top 10,545 differental probes; lower panel: the methylation profile of all available methylation probes used, indicating no significant differences between cases based on all available methylation data. Abbreviation: Frameshift del: Frameshift deletion.

PART IV

General Discussion



CHAPTER 8

General discussion

PART II: SURVEILLANCE OF BARRETT'S ESOPHAGUS

One of the aims of this thesis was to improve surveillance of patients with Barrett's esophagus (BE). Histological assessment is essential for optimal surveillance strategy, and diagnosis of low grade dysplasia (LGD) is important in triaging patients with BE. Until now, LGD diagnosis suffers from low interobserver agreement and low predictive value for progression in general [44]. Therefore, we addressed here the question if the pathological diagnosis of LGD might be improved by the usage of well-defined histological criteria. Furthermore, the predictive value of Cyclin A was assessed in a prospective cohort of patients with BE and compared to a selected group of other well-known biomarkers.

Chapter 3: LGD diagnosis has a central role in in the follow-up of BE-patients. Historically, LGD diagnosis is hampered by a high interobserver variation and its predictive value is questionable [44]. In the last decade studies have shown that expert-confirmed LGD diagnosis has the potential to accurately predict progression [41,45,46,91].

Although it has been shown that expert pathologists with expertise in BE are able to select BE patients at risk of progression, it remains uncertain which histological criteria are most predictive for the LGD diagnosis. The criteria for LGD are not uniformly used by experts. Therefore, 12 different histological criteria were evaluated within a group of pathologists with expertise in BE histology. Those criteria with highest interobserver agreement were selected for additional analysis correlating the presence of the criteria and occurrence of progression on follow-up. Of the 12 histological criteria for LGD, four showed at least a moderate interobserver agreement, namely loss of surface maturation (defined as no maturation of the epithelium seen on low power from the proliferation zone until the surface), mucin depletion (defined as almost total to total disappearance of mucus from the surface columnar cells on high power), nuclear enlargement (defined as a nuclear size at least two times as large as nuclei of the normal not inflamed columnar epithelium) and increase of mitosis (defined as at least one mitosis at the epithelial surface or in the neck of the crypts, mitosis in the base of the crypt are disregarded).

The predictive value of the four criteria were validated using a cohort of patients followed for many years within the Probar study. The Probar study is a Dutch multicenter prospective cohort of patients with newly diagnosed or known BE, who received endoscopic follow-up according to the American College of Gastroenterology [38], until progression to high grade dysplasia (HGD) or esophageal adenocarcinoma (EAC) occurred [117]. From this cohort patients with LGD and progression on follow-up were matched with patients with LGD without progression. The histological slides were examined by expert pathologists using the four specific histological criteria. The presented criteria showed high independent predictive value for progression in a multivariate Cox regression statistical analysis (hazard ratio (HR) of 3.52, 95% CI 1.56-7.97, p=0.003).

This is the first study trying to uniform and standardize histological approach to BE pathology. If well-defined criteria are applied, the overall value of LGD diagnosis increase considerably allowing better selection of patients at risk for progression. The chosen approach should be further tested in independent group of patients with well-defined clinical status and follow-up. If these studies support our initial observation, the histological criteria as suggested by us has the potential to improve the surveillance of patients with BE.

In addition to histology, different biomarkers have been introduced to the BE field. Earlier we were able to study P53 and SOX2 expression by immunohistochemistry in the patients of the Probar cohort and found significant value of both biomarkers for prediction of progression compared to the standard histological evaluation [94,95]. Other groups also stated Cyclin A to be promising biomarker in this context [48,125]. In **chapter 4** Cyclin A was extensively studied in patients from the Probar cohort. Additionally, the incremental value of Cyclin A, compared to P53 and SOX2 was studied using a model which included Cyclin A, P53, SOX2, AMACR and the histological diagnosis. Thereby, P53 showed the highest incremental value followed by SOX2. In contrast, the incremental value of Cyclin A was limited in this analysis (change of the area under the curve 0.003).

The small added value of Cyclin A is not entirely surprising. Cyclin A is expressed in proliferating cells and is considered to be informative of neoplastic progression when surface epithelial BE cells express Cyclin A. However, extensive inflammation and epithelial damage can also lead to increase in mitotic activity and thus luminal expression of Cyclin A. Similar findings were stated for KI67, one of the first promising biomarkers tested in BE, which has been almost entirely disregarded nowadays as a marker for the prediction of progression [56].

Our study shows the importance of validation studies and the need of integrated analysis of biomarkers. Since the number of new biomarkers in the BE field is growing it is not clear which one could actually add to prediction of prognosis compared to those biomarkers that had been shown to be useful in previous extensive studies.

PART III: ESOPHAGEAL ADENOCARCINOMA

In this part of this thesis we studied established EAC. In **chapter 5**, tumor budding (TB) was evaluated in early EAC. In **chapters 6 and 7** expression of P53 and SOX2 was tested in advanced EAC and correlated to the clinico-pathological characteristics.

Our study in **chapter 5** on early EAC has shown that high TB is associated with an increased risk of LNM (odds ratio (OR) 3.5, 95% CI: 1.05-11.68, p-value: 0.041) and tumor related death (HR 2.2, 95% CI:1.17-4.12, p-value: 0.014) in patients with a pT1b EAC.

In contrast to colorectal cancer, TB has not been extensively studied to date in EAC and only limited data is available [147,165,168]. In pT1 adenocarcinoma of the colon, high TB is clearly predictive of LNM [153,154,176,179].

A tumor bud is uniformly defined as a solitary tumor cell or a group of at most 4 or 5 tumor cells, completely surrounded by stroma [144]. The method of counting tumor buds on the other hand vary wildly, and there is no uniform approach on TB evaluation [144]. In this study we compared different methods of TB evaluation and identified the method of Ohike as most informative. In our opinion, TB can add to the risk assessment of LNM in early EAC, similarly to colorectal cancer. According to our results, the method of Ohike is most suitable and might be used for future studies.

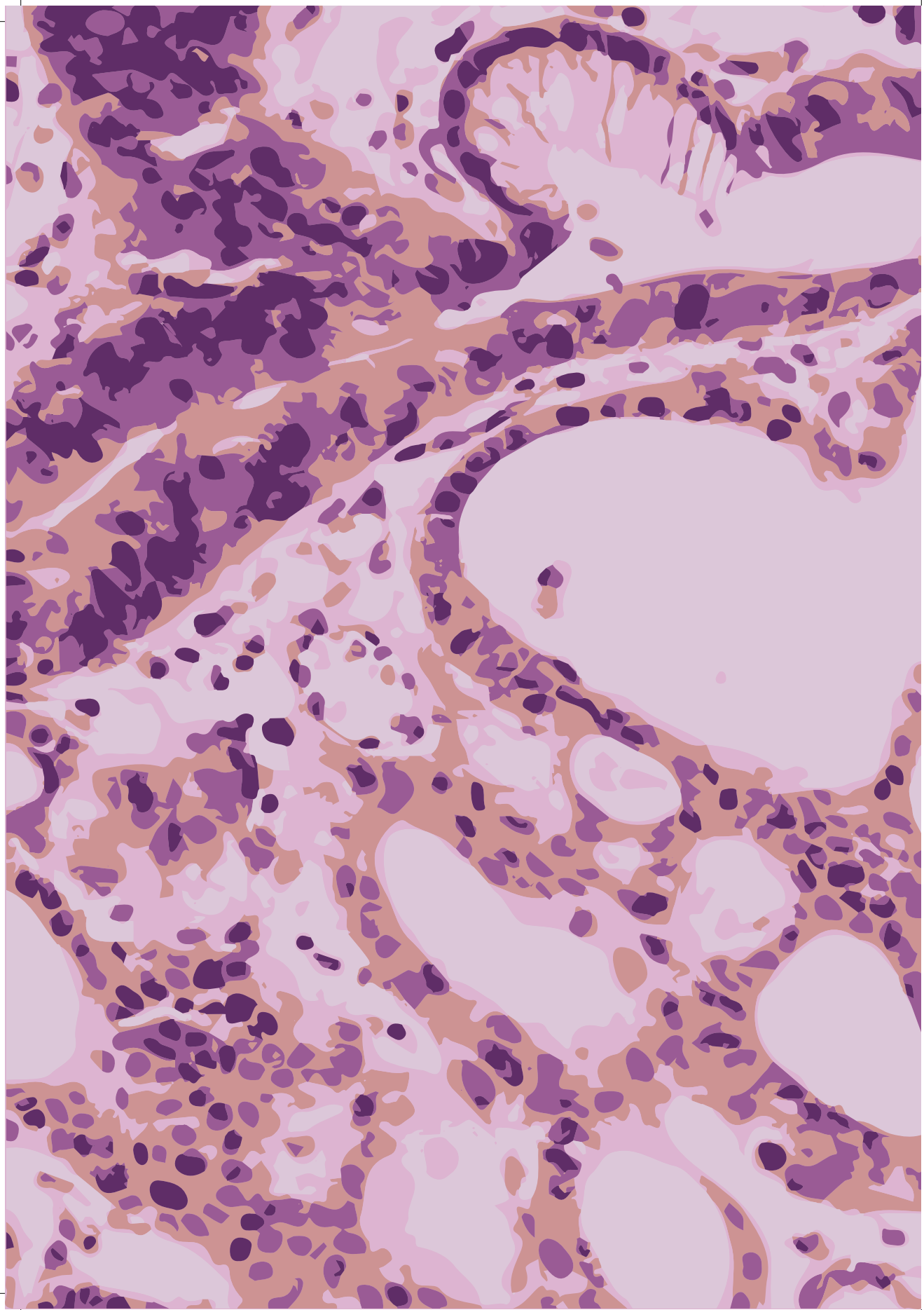
In comparison to early EAC advanced EAC has generally a dismal survival. Presently the only clinically used method of prognostication for these patients is the TNM-staging criteria, based on the resection specimen [75]. Patients with Stage IA disease show a 5-year survival of almost 90% while with an increasing stage the 5-years survival decreases to almost 0% in patients with Stage IV EAC. Although the TNM-staging criteria subdivides the entire group of patients into eight stages with its own survival, the prognosis of an individual patient is still difficult to predict [81]. Therefore biomarker research is ongoing to resolve this problem but the studies performed so far are insufficient to make a firm conclusion. Most studies included patients with adenocarcinomas as well as squamous cell carcinomas. Also, patients were exposed to different treatment protocols.

In **chapter 6** the prognostic value of SOX2 was studied in two independent cohorts of patients with EAC (Rotterdam cohort and OCCAMS cohort). Loss of SOX2 expression proved to be predictive of adverse outcome in both of these cohorts of patients, especially in patients with pN0 and Stage I.

The function of SOX2 is not clarified at this moment. SOX2 is linked to gastric and intestinal differentiation and is expressed in the majority of patients with non-dysplastic BE. During the progression of BE to EAC, SOX2 is increasingly lost, probably as a result of loss of epithelial differentiation [95]. In gastric carcinoma SOX2 acts as a tumor suppressor gene [61] and our result support similar functions for EAC.

Another biomarker for which conflicting results are published is P53. In **chapter 7** the prognostic value of P53 was evaluated in a large, well defined cohort of patients with advanced EAC and treated by surgically resection as a single modality. Aberrant expression of P53 was predictive of adverse OS and DFS.

P53 has a central role in the progression from non-dysplastic BE to EAC and *TP53*, the coding gene of this protein, is considered to be one of the driving genes for malignant progression. This is substantiated by whole genome sequencing studies which found a high mutation frequency in *TP53* [24-27]. However, for the value of P53 for prognostication in EAC was under debate, due to the methodological problems in earlier research. Patients with different treatment modalities were included in those studies[82,83,215] and different cut-offs for P53 expression were applied [82,83,215]. Our study is the first one to circumvent these problems by usage of an uniform patient group and standardized evaluation of P53. Also, we showed that expression pattern of P53 correlates with the genetic status of *TP53* and correlated with the genome methylation pattern. This observation might have therapeutic consequences in the future. Presently medications are being developed which are intended to restore the wild-type activity of P53, one of the most promising compounds in these is APR-246 [241]. Additionally, the response of EAC on chemotherapy might be influenced by *TP53* mutations [202,235]. Clinical trials, which stratified patients based on the *TP53* mutations are being implemented and some completed their recruitment of patients [218]. If *TP53* mutational status is predictive for the response on chemotherapy and patients are being stratified based on their *TP53* mutations, it might be cost-effective and less time consuming to perform P53 immunohistochemistry first and only when a heterogeneous expression is detected, to perform next-generation mutational analysis.



CHAPTER 9

Concluding remarks and future prospects

Patients with BE are at risk of developing EAC, especially when dysplasia has been discovered. With an increasing grade of dysplasia the chance of malignant progression increases. Therefor patients, in which LGD has been diagnosed, are subjected to a stringent follow-up protocol and is eradication considered in some of these patients. But the progression rate of these patients is still low and the interobserver variation is considerable.

For a better selection of patients with LGD at risk of progression the histological criteria for LGD were refined and the interobserver variation of each criterion was calculated. Four criteria proved to have a moderate to good interobserver variation, namely loss of maturation, mucin depletion, nuclear enlargement, and increase of mitosis. In patients in which more than one of these criteria present in one biopsy are at increased risk for progression to HGD or EAC in a discovery cohort as well as a validation cohort. The combination of these criteria with aberrant immunohistochemical staining of P53 showed considerable overlap, and the patients with more than one criterion present and aberrant P53 expression showed the highest portion of progressions while patient with either more than one criterion or aberrant P53 staining showed an intermediate risk of progression.

To further improve the selection of BE patients at risk of progression another immunohistochemical biomarker, Cyclin A, was tested in 720 patients with all grades of BE and showed to be correlated with progression with a relative risk of 2.4. Furthermore the incremental value of Cyclin A was calculated in a set of biomarkers consisting of P53, SOX2 and Cyclin A in combination with the histological diagnosis. Although overexpression of Cyclin A is correlated with progression it showed the least incremental value in this panel of biomarkers.

Although it should be tested in an independent prospective cohort of BE patients, preferably scored by general pathologists, these results show that patients with either aberrant expression of P53 or more than one of the four histological criteria should enter a stringent follow-up protocol while eradication should be considered in patients with both more than one of the four histological criteria and aberrant expression of P53. While in patients without aberrant P53 and none or one of the histological criteria a follow-up protocol as indefinite for dysplasia could be considered. The addition of Cyclin A to this panel does not improve the prognostic value and therefore could be omitted.

If BE progresses to EAC and this is discovered in an early stage, in which the tumor has not extended into the muscularis propria, patients can be treated with a local resection provided that the chance of LNM is low. Histological and clinical criteria are used to estimate the chance of LNM.

A relatively new histologic criterion thoroughly tested in other solid tumors is TB. In EAC TB is predictive for LNM in patients with a pT1b tumor in a discovery cohort as well as a validation cohort with a OR of 3.5. Not only is TB associated with LNM, it is also prognostic for OS and DFS. Therefor TB should be stated for patients with a pT1b EAC. Although the

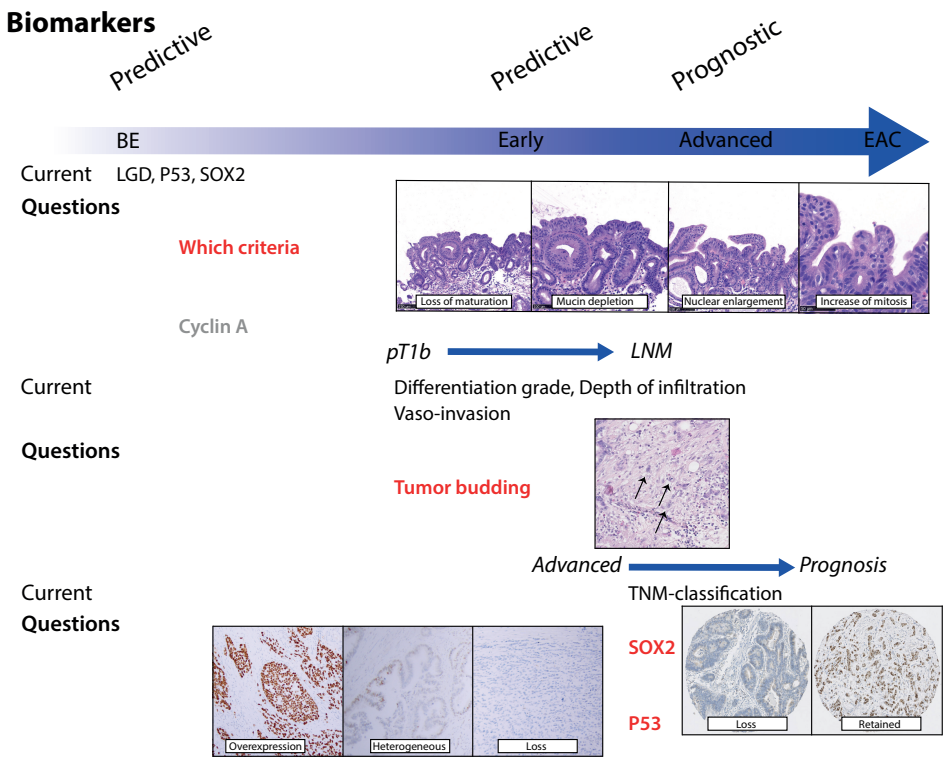


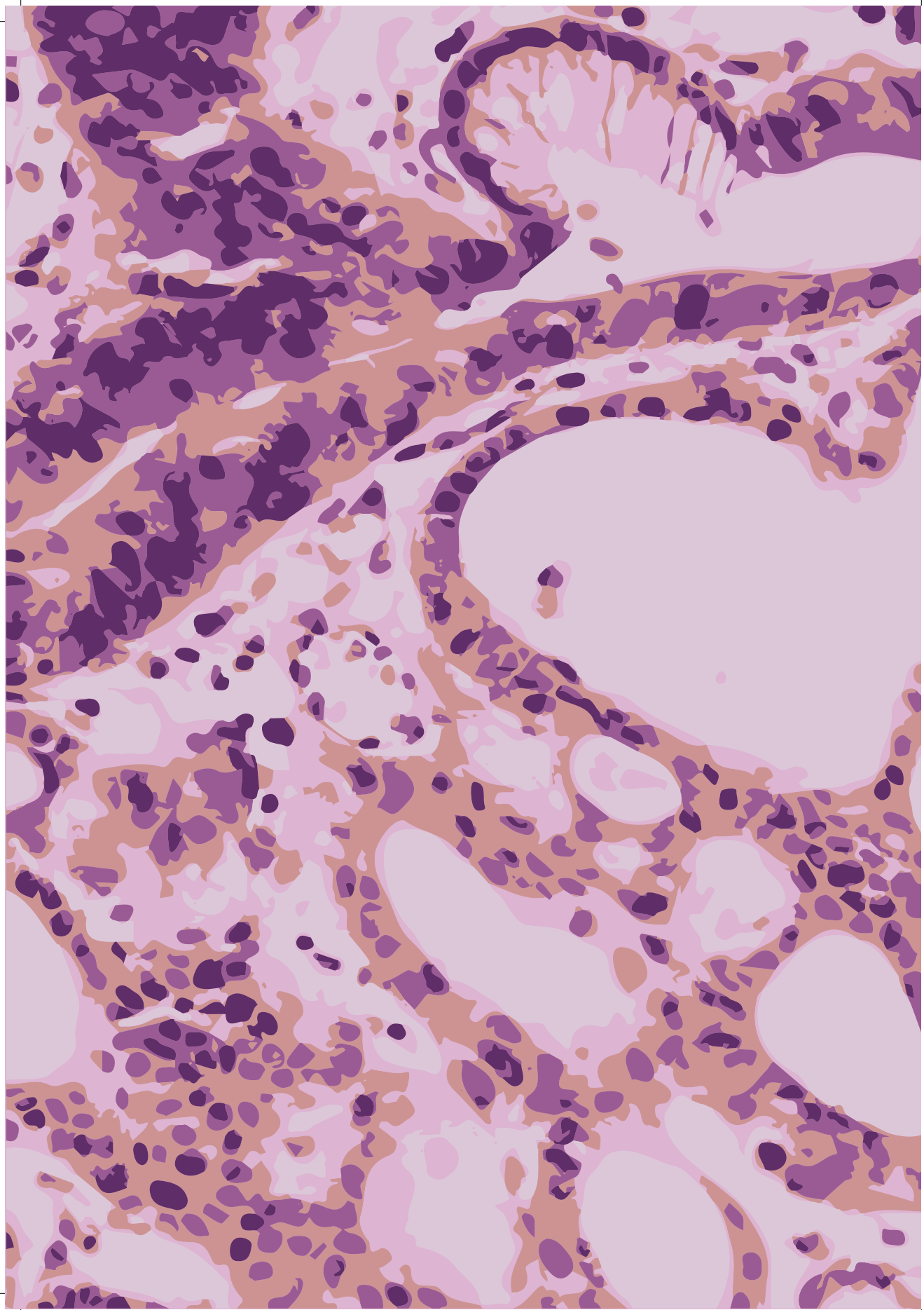
FIGURE 1: Schematic overview of this thesis. In Part II we have shown four specific histological criteria for low grade dysplasia (LGD) to have a low interobserver variation and to be predictive for progression of Barrett's esophagus (BE) to esophageal adenocarcinoma (EAC). Although Cyclin A is independently predictive for progression in BE, the additional value for prediction of progression in comparison to LGD, P53 and SOX2 is neglectable, and could be omitted. Tumor budding has been shown to be predictive for lymph node metastasis (LNM) in patients with early EAC. In patients with advanced EAC SOX2 and P53 are of prognostic value, next to the tumor node metastasis (TNM) classification.

results should be confirmed in a large prospective trail, patients with a high TB are at risk for LNM and a radical esophagectomy with neoadjuvant treatment should be considered instead of a local resection.

Prognostication of patients with EAC is nowadays primarily based on the depth of tumor infiltration and the presence, location and number of LNM and distant metastasis. To further improve the prognostication of these patients, two biomarkers were tested. Loss of SOX2 proved to be prognostic in patients with EAC, especially in patients with Stage I disease. The use of this marker in addition to the current staging systems could be of relevance, especially in these selected patients.

The second biomarker tested is P53, which proved to be prognostic in patients with EAC for OS as well as DFS. Moreover, it has been shown that the immunohistochemical aberrant expression is predictive for the type of mutation in *TP53*, while patients with a normal P53 expression could harbor *TP53* mutations. Our results could suggest different pathways of pathogenesis related to the P53 pathway, with downstream additional mutations of other genes and methylation alterations. To further reveal genetic profiles which correlate to prognostic signatures of EAC further work has to be performed, as well as to elucidate the precise biological role of P53 and SOX2 in the development and biology of EAC, possibly revealing new treatment possibilities.

SUMMARY / NEDERLANDSE SAMENVATTING



CHAPTER 10

Summary

Barrett's esophagus (BE) is the only known precursor lesion of esophageal adenocarcinoma (EAC). Patients with BE are at risk for developing EAC, with an incidence estimated at 0.1-0.4% yearly. The transition of nondysplastic BE (NDBE) to EAC is thought to be a gradual process in which the epithelium first shows low grade dysplasia (LGD) followed by high grade dysplasia (HGD). Since patients with advanced EAC have a poor 5-year survival, patients with BE are recommended to undergo follow-up for the detection of HGD or early EAC, so patients can hopefully be treated in an early stage.

Patients with LGD are at increased risk for progression and thus undergo an intensified follow-up scheme or in some instances local treatment of the Barrett segment. The diagnosis of LGD is problematic though. The predictive value of LGD is highly variable and the interobserver variation is high. Improved pathological characterization of LGD and the addition of independent biomarkers would help to improve prediction of progression in patients with BE.

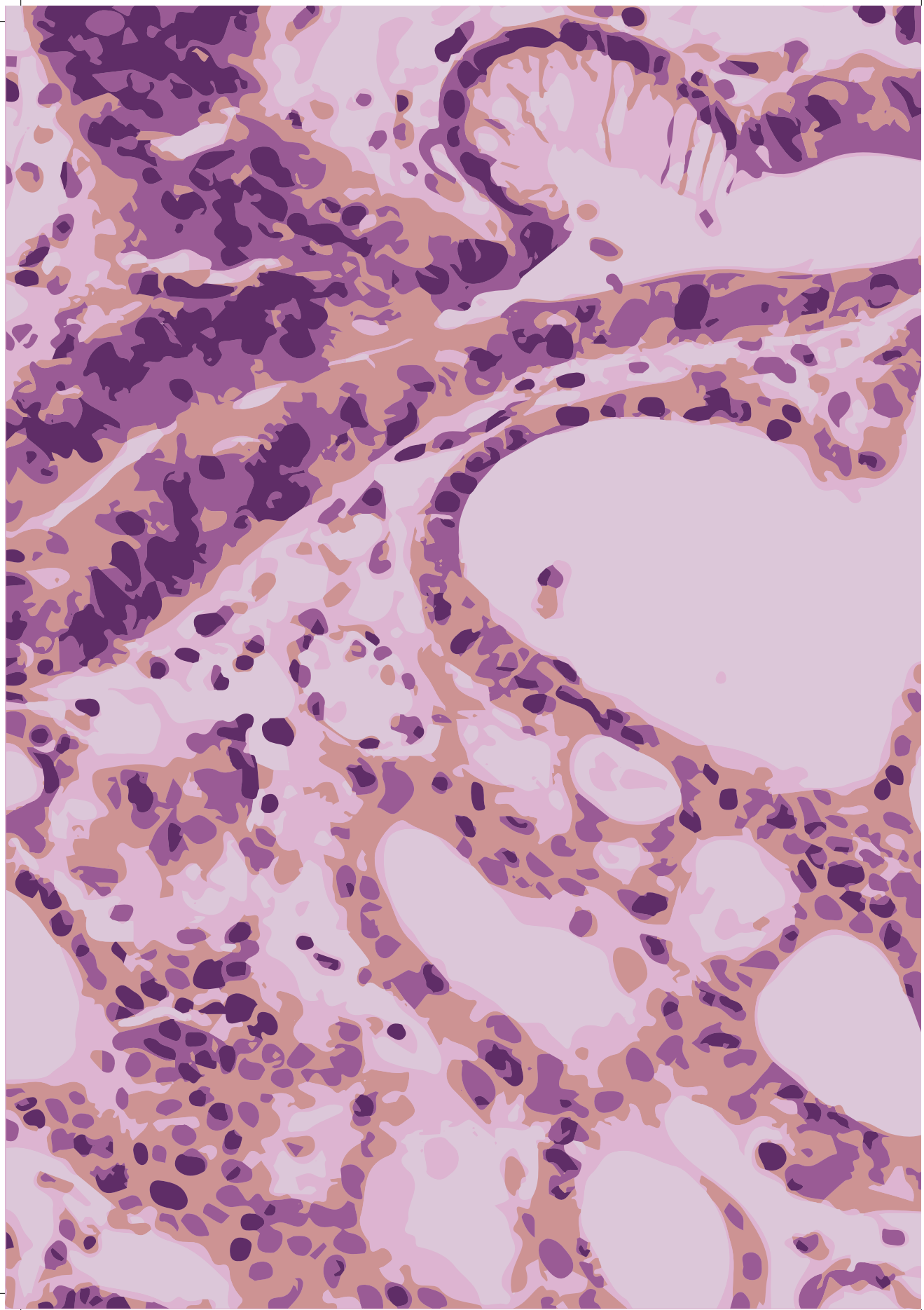
In the second part of this thesis (**chapter 3**) we performed a histological in depth analysis of LGD. Various histological criteria were individually evaluated by four expert GI-pathologists. 12 different LGD criteria were analyzed in two independent groups of patients with known outcome. First of all, four criteria with good interobserver agreement were identified, including loss of maturation, mucin depletion, enlarged nuclei and increase in mitosis. Presence of these changes was significantly associated with outcome in the primary patient group. These results were validated and confirmed in independent BE patients. Combination of these four histological changes and status of P53 expression further improved prediction of progression.

In **chapter 4** the predictive value of Cyclin A was tested in a cohort of 720 prospectively followed patients with BE. Because of the lack of studies in BE combining multiple immunohistochemical markers, Cyclin A was combined with AMACR, P53 and SOX2 results of earlier published data with the goal to select the most predictive markers for progression. Expression of Cyclin A at the luminal side of the biopsy was associated with a two times higher chance of progression to HGD or EAC. When combined in a fully adjusted model, aberrant expression of P53 showed the greatest value, followed by SOX2, Cyclin A and AMACR. The additional value of Cyclin A compared to P53 and SOX2 was minimal.

In the third part of this thesis the predictive and prognostic value of biomarkers in early and advanced EAC were studied. In **chapter 5** tumor budding (TB) was analyzed in pT1b EAC and was correlated to lymph node metastasis (LNM). Various TB methods were assessed and shown to be predictive of LNM independently of other histological parameters. The usage of immunohistochemistry to improve the visibility of tumor buds was not associated with status of LNM.

Patients with advanced EAC are not eligible for endoscopic resection and mostly treated by neo-adjuvant chemo-radiation therapy and radical esophagectomy. For the

prognostication of these patients the only clinically available method is presently the TNM classification. In **chapter 6** the prognostic value of SOX2 was assessed in two independent cohorts consisting of in total 756 patients treated by radical esophagectomy. Patients with loss of SOX2 showed a significantly shorter median survival in both cohorts of patients of respectively 19.5 and 15.0 months compared to 32.9 and 26.0 months in tumors with retained SOX2 expression. Furthermore, loss of SOX2 was independently predictive for a worse overall survival (OS) with a hazard ratio (HR) of 1.4. Especially in chemotherapy naïve patients with a Stage I tumors loss of SOX2 was predictive for OS with a HR of 3.2.
Finally, in **chapter 7** the prognostic value of P53 in advanced EAC was assessed in resections of 204 chemotherapy naïve patients. Patients with normal expression of P53 showed better OS and DFS compared to aberrant expression. When comparing the immunohistochemical expression of P53 with the *TP53* mutational status we found that most EAC with loss of expression and overexpression showed mutated *TP53* (respectively non-sense mutations and missense mutations). In addition, P53 expression correlated with the global methylation pattern. These findings may be interesting to validate in further studies, since they could reflect different pathogenic pathways in BE, and possibly even an impact of clinical behavior as well as selection of optimal treatment.
In conclusion, this thesis shows that pathological approach to BE histology could improve by the usage of specific histological criteria for LGD. The data presented demonstrate increased predictive value of histological evaluation after the adoption of the suggested criteria. Although these findings are consistent in the primary and validation cohort they should be confirmed in the futures studies to insure reliability. Usage of P53 further increases predictive value of the histological diagnosis. In contrast, additional value of Cyclin A was limited in the integrated study using several biomarkers. Therefore in the pathological practice Cyclin A is not clinically applicable biomarker for progression in patients with BE. In patients with EAC infiltrating into the submucosa TB could be a suitable biomarker of the LNM status. Furthermore, P53 and SOX2 expression in advanced EAC are both independently predictive of outcome. The biological role of both genes and their role in clinical pre-treatment evaluation should be established in further clinical and fundamental studies.



CHAPTER 11

Samenvatting

De ziekte waarbij het normale bekledende plaveisel epitheel van de slokdarm wordt vervangen door maag- of colon-type epitheel, wordt Barrett oesofagus (BE) genoemd. BE is de enige bekende voorloper van slokdarm adenocarcinoom (EAC). Patiënten met BE hebben ieder jaar ongeveer 0.1-0.4% kans op het ontwikkelen van EAC. De verandering van niet-dysplastisch BE (NDBE) naar EAC wordt beschouwd als een gradueel proces waarbij eerst laaggradige dysplasie (LGD) en daarna hooggradige dysplasie (HGD) ontstaat om uiteindelijk te resulteren in EAC. EAC is een tumor met een slechte overleving waarbij ongeveer 50% van de in opzet curatief behandelde patiënten binnen 5 jaar overlijden aan deze ziekte. Om EAC in een vroeg stadium te ontdekken, waarbij curatieve therapie mogelijk is, worden alle patiënten met BE een vervolg traject aangeboden waarbij regelmatig endoscopisch onderzoek wordt verricht.

De histologische diagnose LGD wordt gezien als de eerste stap in de maligne progressie. Patiënten met LGD hebben een verhoogde kans op maligne progressie en worden daarom vaker uitgenodigd voor een endoscopie of, in geselecteerde gevallen, wordt BE lokaal behandeld met radiofrequente ablatie. De histologische diagnose LGD is echter problematisch door de grote variatie tussen beoordelaars en weinig betrouwbare voorspellende waarde. Door de toevoeging van extra methoden om het risico van progressie te voorspellen, kan de voorspellende waarde worden verhoogd en daarmee kunnen de patiënten met de grootste kans op progressie worden geselecteerd.

Het tweede deel van dit proefschrift heeft als doel om de voorspellende waarde van LGD in BE te verbeteren door het toevoegen van biomarkers om de patiënten te selecteren met een verhoogd risico op progressie naar HGD en EAC.

In **hoofdstuk 3** zijn 12 criteria voor LGD onafhankelijk van elkaar gescoord door vier in gastro-enterologie gespecialiseerde (GI) pathologen. Vier van de 12 criteria, te weten verlies van uitrijping naar het oppervlak, verlies van slijmproductie, vergrootte kernen en toename van delingen tonen een goede overeenkomst tussen beoordelaars. Als meer dan één van deze vier criteria aanwezig zijn in een biopt met LGD, heeft de patiënt 3,5 keer zoveel kans op progressie naar HGD of EAC in vergelijking met biopten met geen of één van deze criteria.

Met de toevoeging van de immunohistochemische expressie van P53 aan de histologische criteria toonden patiënten met zowel een afwijkende P53 alsook positieve histologische criteria de hoogste kans op progressie naar HGD of EAC.

In **hoofdstuk 4** hebben wij de toegevoegde waarde van Cycline A getest in een cohort van 720 prospectief gevolgde patiënten met BE. Omdat er een gebrek is aan onderzoek in BE welke meerdere biomarkers combineren om de meest voorspellende combinatie te vinden, werden de resultaten van Cycline A gecombineerd met AMACR, P53 en SOX2. Patiënten met een verhoogde expressie van Cycline A aan het oppervlak van het biopt, hadden twee keer meer kans op progressie naar HGD of EAC in vergelijking met patiënten met een

normale expressie van Cycline A. In een statistisch model waarbij deze resultaten werden gecombineerd met bovengenoemde biomarkers, toonde P53 de grootste verandering in het oppervlak onder de curve (AUC) (0.050). Verlies van SOX2 en de histologische diagnose LGD toonde hierna de meeste verandering in de AUC, waarbij Cycline A en AMACR zeer weinig toevoegde. De combinatie van histologische diagnose LGD, afwijkende P53 expressie en verlies van SOX2 expressie toonde de hoogste voorspellende waarde (AUC: 0.72; 95% betrouwbaarheid interval 0.67 – 0.77).

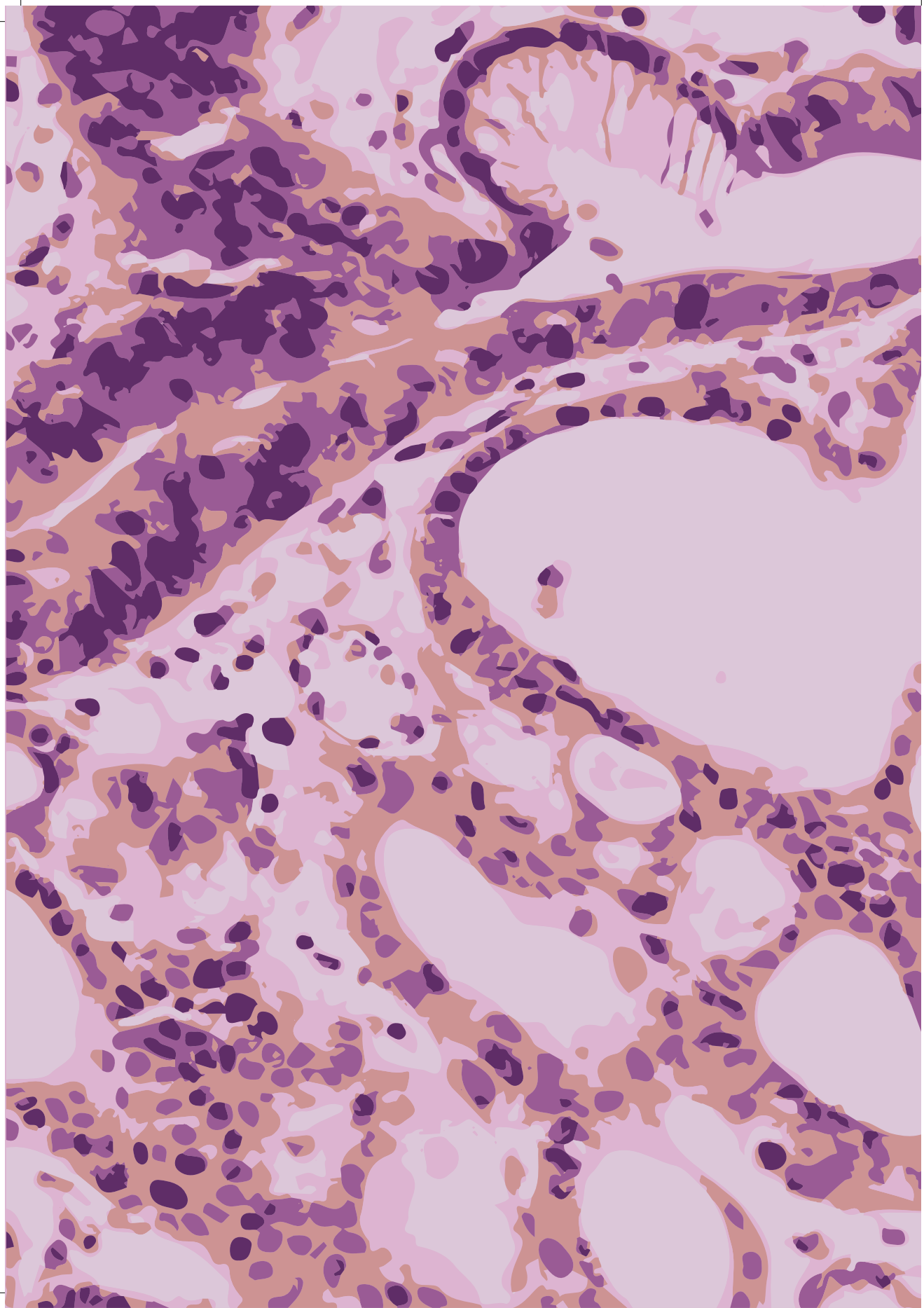
Het derde deel van dit proefschrift heeft zich toegespitst op de voorspellende waarde van biomarkers in EAC. Vooralsnog raden de richtlijnen aan om patiënten met EAC, welke curatief behandeld kunnen worden, een resectie te laten ondergaan. Als het nog een oppervlakkig EAC betreft kan een endoscopische resectie worden overwogen. Een voorwaarde hiervoor is echter dat de kans op lymfklier uitzaaiingen (LNM) zeer klein is, omdat met een endoscopische resectie deze niet behandeld worden. Momenteel zijn er meerdere tumor karakteristieken bekend die de kans op LNM vergroten, onder andere differentiatie graad, invasie diepte van de tumor en invasie van de tumor in vaten. In **hoofdstuk 5** wordt nog een tumor karakteristiek gecorreleerd aan LNM, namelijk tumor budding (TB). In dit hoofdstuk werden drie beschreven methoden van het beoordelen van TB vergeleken voor hun voorspellende waarde voor LNM. De resultaten laten ziet dat methode van Ohike het meest voorspellend was, waarbij patiënten met veel TB een 3,5 keer hogere kans op LNM hebben. De TB beoordeeld op de met pankeratine gekleurde coupe, om beter de TB zichtbaar te maken, was niet informatief voor LNM predictie.

Patiënten met een vergevorderde EAC kunnen niet meer curatief behandeld worden met een endoscopische resectie en ondergaan radicale slokdarmresectie met voorafgaand behandeling met chemo- en radiatie therapie. Om de overleving van deze patiënten te voorspellen wordt op dit moment alleen de TNM-classificatie, gebaseerd op de diepte van de tumor invasie, aanwezigheid, aantal en locatie van LNM en uitzaaiingen op afstand, in de kliniek gebruikt.

In **hoofdstuk 6** wordt de voorspellende waarde van SOX2 voor overleving beoordeeld in radicale slokdarmresecties van twee onafhankelijke cohorten van in totaal 756 patiënten. Patiënten met verlies van SOX2 expressie hebben een significante kortere mediane overlevingstijd in allebei de cohorten van respectievelijk 19,5 en 15,0 maanden, vergeleken met tumoren met behoud van SOX2 (mediane overlevingstijd van respectievelijk 32.9 en 26.0 maanden). Tevens is verlies van SOX2 expressie een onafhankelijke voorspeller voor de totale overlevingsduur (OS), met een risico verhouding (HR) van 1.4. In patiënten met een Stadium I EAC zonder voorbehandeling met chemotherapie was SOX2 het meest voorspellend voor OS met een HR van 3.2.

In **hoofdstuk 7** wordt P53 expressie in 204 patiënten zonder chemotherapie behandeling beschreven. Tevens wordt de immunohistochemisch expressie van P53 vergeleken met

de aanwezige mutaties in het *TP53* gen en het methylatie profiel van de tumor. Patiënten met een heterogene expressie van P53 in de EAC toonden een betere OS en ziekte vrije overleving vergeleken met aberrante expressie van P53. De mutaties in het *TP53* gen waren geassocieerd met het expressie patroon van P53 (sterke expressie of verlies van expressie). Tevens werd er een correlatie tussen P53 expressie en methylatie status gevonden.
Concluderend, patiënten met BE worden uitgenodigd voor regelmatig onderzoek om progressie aan te tonen in een vroeg en curatief stadium. Om te voorspellen welke patiënten progressie gaan vertonen, kan een immunohistochemisch panel, bestaande uit P53 en SOX2, worden gebruikt tezamen met de histologische diagnose LGD. In patiënten met LGD moet de aanwezigheid van de vier boven beschreven histologische criteria worden aangeduid, waarmee de voorspelbaarheid voor progressie wordt verhoogd. Met deze informatie zou besloten kunnen worden om de BE in het stadium van LGD te behandelen om progressie te voorkomen. Patiënten die progressie tonen naar EAC, welke infiltreert tot maximaal in de submucosa, moet de aanwezigheid van TB worden beoordeeld om beter de aanwezigheid van LNM te voorspellen, waarmee de behandeling aangepast kan worden. P53 en SOX2 zijn niet alleen voorspellend in BE maar ook voorspellend voor overleving in EAC. Tevens is de immunohistochemische expressie van P53 gecorreleerd aan de mutaties in het *TP53* gen als ook methylatie status.



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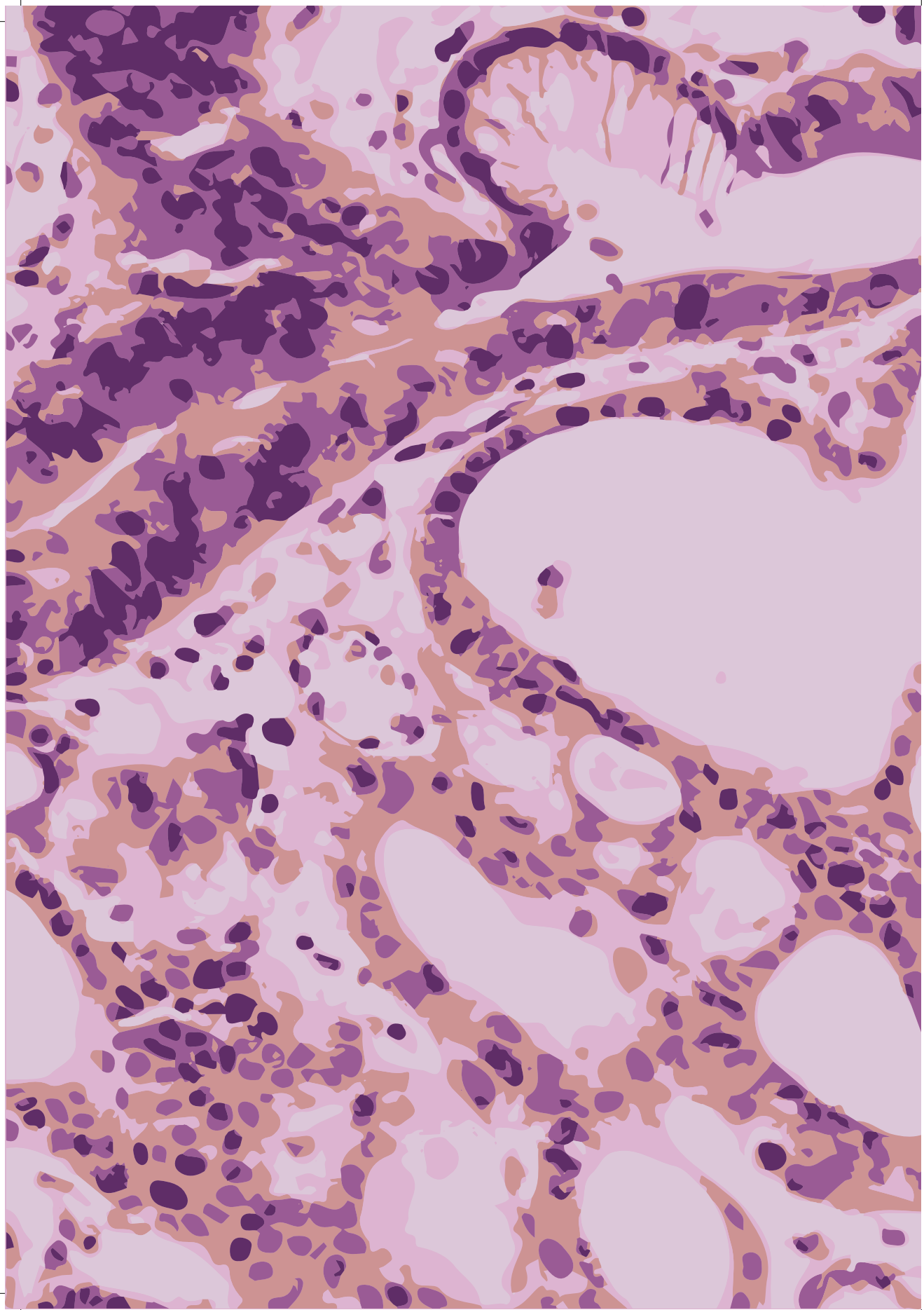

APPENDICES

CURRICULUM VITAE

Fiebo ten Kate werd geboren op 27 juli 1980 te Bergambacht. Na het behalen van het atheneum aan het RSG Rotterdamsch Lyceum te Rotterdam in 1999 werd hij uitgeloot voor de studie Geneeskunde en is begonnen aan de studie Medische Informatiekunde aan de Universiteit van Amsterdam, alwaar hij zijn propedeuse haalde in 2000. In dat jaar werd Fiebo ingeloot voor de studie Geneeskunde aan de Rijks Universiteit Groningen, alwaar hij zijn doctoraal en artsdiploma behaalde in december 2007. Na behalen van zijn artsexamen heeft Fiebo als arts-assistent niet in opleiding opeen volgend gewerkt op de afdeling Urologie in het Academisch Medisch Centrum te Amsterdam, de afdeling Chirurgie in de Diakonessenhuis te Utrecht en op de afdeling Spoedeisende hulp in het Gemini ziekenhuis in Den Helder. In oktober 2010 begon Fiebo aan zijn opleiding tot patholoog aan het Erasmus Medisch Centrum in Rotterdam. Gedurende zijn opleiding werd een onderzoekstage verricht bij Katharina Biermann, wat na het beëindigen van zijn opleiding in 2015 werd voortgezet in een promotieonderzoek, onder supervisie van Prof. Dr. L.H.J. Looijenga en Prof. Dr. F. van Kemenade, gecombineerd met een Fellowship Gastro-Intestinale en Lever pathologie aan het Erasmus Medisch Centrum. Na het beëindigen van dit fellowship in december 2017, heeft Fiebo waargenomen op de afdeling Pathologie aan het Canisius Wilhelmina Ziekenhuis te Nijmegen, waarna hij sinds mei 2018 werkzaam is als patholoog bij het Laboratorium Pathologie Oost Nederland.

LIST OF PUBLICATIONS

Value of Cyclin A immunohistochemistry for cancer risk stratification in Barrett esophagus surveillance: A multicenter case-control study.
SH van Olphen, **FJC ten Kate**, M Doukas, F Kastelein, EW Steyerberg, HA Stoop, MC Spaander, LH Looijenga, MJ Bruno, K Biermann; ProBar-Study Group. Medicine; 2016: November; 95(47):e5402.

Loss of SRY-box2 (SOX2) expression predicts adverse survival of patients with oesophageal adenocarcinoma.
F.J.C. ten Kate, S.H. van Olphen, M. Bruno, B.P.L. Wijnhoven, J.J.B. van Lanschot, L.H.J. Looijenga, R.C. Fitzgerald, K. Biermann. British Journal of Surgery; 2017: September; 104(10):1327-1337

Endoscopically resectable T1 colorectal carcinomas in four rounds of fecal immunochemical test-based colorectal cancer screening
E. Wieten, E. J. Grobbee, P. Didden, K. Biermann, **F. J.C. ten Kate**, A. D. Koch, E. J. Kuipers, M. J. Bruno, M.C.W. Spaander, Submitted for publication

Pattern of p53 protein expression is predictive for survival in chemoradiotherapy-naïve esophageal adenocarcinoma.
F.J.C. ten Kate, L. Suzuki, L.C.J. Dorssers**,** W.N.M. Dinjens, D.T.W. Jones, D. Nieboer, M. Doukas , J.J.B. van Lanschot, B.P.L. Wijnhoven, L.H.J. Looijenga, K. Biermann, Oncotarget; 2017: October; 24; 8(61):104123-104135

Improved progression prediction in Barrett's esophagus with low grade dysplasia using specific histological criteria
F.J.C. ten Kate, D. Nieboer, F.J.W. ten Kate, M. Doukas, M.J. Bruno, M.C.W. Spaander, L.H.J. Looijenga, K. Biermann, on behalf of the Probar-study group and Palga Group. American Journal of Surgical Pathology; 2018: July; 42 (7):918-926

Tumor budding is predictive for lymph node metastasis and survival in patients with pT1b esophageal adenocarcinoma
F.J.C. ten Kate, A.W. Gotink, M. Doukas, D. Nieboer, B.P.L. Wijnhoven, J.J.B. van Lanschot, L.H.J. Looijenga, A.D. Koch, K. Biermann, on behalf of the SubLyme group, Submitted

Do pathologists agree with each other on the histological assessment of pT1b esophageal adenocarcinoma?
A.W. Gotink, **F.J.C. ten Kate**, M. Doukas, B.P.L. Wijnhoven, M.J. Bruno, L.H.J. Looijenga, A.D. Koch, K. Biermann. United European Gastroenterology Journal; Accepted for publication

PHD PORTOFOLIO

Name PhD student: F.J.C. ten Kate
PhD period: 01-10-2015 until 31-12-2017
Erasmus MC Department: Pathology
Promotors: Prof. Dr. L.H.J. Looijenga and Prof. Dr. F.J. van Kemenade
Research School: Molecular Medicine, Erasmus MC
Supervisor: Dr. K. Biermann

PhD training	Year	ECTS
General courses		
Workshop Presenting Skills for Scientists	2016	1,4
Survival Analysis Course 23-24 June	2016	0,5
Photoshop and Illustrator	2016	0,3
Course on R	2017	1,8
Specific courses		
Basiscursus Oncologie	2016	1
NGS in DNA Diagnostics Course	2016	1
Seminars and workshops		
JNI meeting	2015-2017	1
PALMS meeting	2015-2017	1
LEPO meeting	2015-2017	1
19th Molecular Medicine Day	2015	0.3
20th Molecular Medicine Day	2016	0.3
Oral presentations		
European Society of Pathology	2016	1
Gastroenterologie dagen	2016	1
European Society of Pathology	2017	1
PALMS meeting	2015-2017	1
LEPO meeting	2015-2017	1
JNI meeting	2015-2017	1
Poster presentations		
Pathologen dagen	2015	1
20th Molecular medicine day	2016	1
Pathologen dagen	2016	1
Digestive Disease Week	2016	1
European Society of Pathology (2x)	2017	1

PhD training	Year	ECTS
International conferences		
Pathologen dagen	2015-2016	0
Gastrointestinal, Liver and Pancreatic Pathology	2015	1
Digestive Disease Week	2016	1
European Society of Pathology	2016	1
Gastroenterologie dagen	2016	0
Symposium on upper GI and pancreatobiliary pathology	2016	0.2
European Society of Pathology	2017	1
Teaching		
Supervision J Henriquez	2016-2017	4
Supervision AIOS Pathology	2015-2017	4
Teaching students medicine	2015-2017	2.4
Presentation WICH	2016	1

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