Biological and Clinical Parameters to Improve Outcome in Oesophageal Cancer

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Colofon

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Chapter one

General introduction and outline of the thesis

E.L.A. Toxopeus

Oesophageal cancer is the ninth most common malignancy worldwide and its incidence is rising, with annually more than 450.000 new patients worldwide [1–3]. Approximately 2500 patients are diagnosed each year in The Netherlands [4]. Oesophageal cancer can be divided into two main histological subtypes: squamous cell carcinoma (SCC) and adenocarcinoma (AC). Risk factors for SCC are cigarette smoking and alcohol consumption. Gastro-oesophageal reflux disease and obesity are the main risk factors for AC [5]. It is known that high-income countries have higher incidences of AC and also men are at a greater risk for AC [6]. The prognosis of oesophageal cancer is poor with a five-year overall survival rate of less than 20% [7–9]. Oesophageal cancer ranks sixth in mortality among all cancers 10. At the time of diagnosis, more than 40% of patients have already disseminated disease. Hence, only the minority of patients can be offered treatment with curative intent. In patients with locoregional disease (cT1-4aN1-3M0), multimodal treatment is applied and five-year survival rate may be as high as 50 % [2,9,11,12].

Surgical resection of the oesophagus with the locoregional lymph nodes is the treatment of choice for most patients [8,9,13]. Over the past two decades, survival after surgery has improved by the introduction of neoadjuvant and adjuvant treatments including chemotherapy, radiotherapy and a combination of both 2. In the Netherlands, neoadjuvant chemoradiotherapy followed by surgery is a standard of care [9,11,12]. Neoadjuvant chemoradiotherapy according to CROSS consists of paclitaxel and carboplatin and concomitant 41.4 Gy radiotherapy. This treatment induces tumour down staging, increases the rate of a complete resection of the tumour and is associated with a lower recurrence rate and better overall survival. There is a clinically relevant difference in response to chemoradiotherapy between SCC and AC. SCC show more frequent a complete pathological response compared to AC [8,9].

Prognostic or predictive parameters including biomarkers may help in selecting patients for treatments and could give valuable information on long-term survival after treatment [14]. Genetic ad epigenetic aberrations in the tumors including microRNA's are being investigated to improve diagnostics, treatment and outcome. Not only genetic aberrations detected in tumour tissues but also the presence of circulating DNA or tumour cells in the bloodstream of patients with cancer may change the landscape of cancer care in next few years [15,16].

AIM OF THE THESIS

Response to treatment varies widely per patient. Some tumors ideally, a patient-tailored treatment should be available to optimise treatment, minimise side-effects and to improve survival. This thesis focuses on biological and clinical predictive and prognostic parameters in oesophageal cancer in patients that underwent surgery alone, neoadjuvant chemoradiotherapy and induction chemotherapy followed by surgery and palliative chemotherapy.

OUTLINE OF THE THESIS

The thesis starts with a review entitled "New therapeutic strategies for squamous cell cancer and adenocarcinoma" (**chapter 2**). This chapter gives an overview of neoadjuvant treatment strategies. The optimal preoperative treatment, prognostic and predictive effects of single nucleotide polymorphisms, the role of transtuzumab, oesophagectomy after neoadjuvant treatment, if para-aortic lymfadenectomy should be performed in junctional tumours and the transhiatal oesophagectomy are elucidated.

PART I - POTENTIAL BIOMARKERS

Biomarkers are defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[17]. In cancer, biomarkers can be used as detectors of disease, therapeutic and prognostic markers. In oesophageal cancer a variety of biomarkers have been identified and recently correlated to the hallmarks of cancer for oesophageal adenocarcinoma [18].

MicroRNAs are small non-coding RNAs which function is to regulate gene expression. Approximately one-third of all human genes are directly regulated by microRNAs [19,20]. MicroRNAs, by interaction with complementary sequence in mRNA, cause inhibition of post transcriptional translation of induces targeted mRNA degradation [21,22]. Several microRNAs that may have a role in cancer initiation and progression have been now identified, including microRNA-126. It is thought that microRNAs play an important role in cancer biology of different cancer types including breast-, gastric-, and pancreatic neoplasms [23]. In **chapter 3** microRNA-126 was studied in relation to tumour cell viability and survival in patients with AC. **Chapter 4** describes the expression of c-MET, the hepatocyte growth factor receptor, in relation to survival of patients after surgery. C-MET is a target for new chemotherapeutic agents. Targeting c-MET is likely most useful in patients that show overexpression of c-MET in the tumour [24].
The most important serum biomarkers of liver injury are aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), of which the latter has been linked to response to neoadjuvant chemotherapy in esophageal carcinoma, however squamous cell carcinoma [25]. **Chapter 5** investigates whether serum liver function biomarkers ASAT, ALAT, albumin, CRP, gGT, total bilirubin and ALP are associated with response to chemoradiotherapy and survival in patients with AC.

PART II – MULTIMODALITY TREATMENT

Multimodality treatment is treatment of choice for patients with oesophageal cancer. To evaluate which patient responds best to a given therapy, a nomogram can be useful. A nomogram, established in 1884, includes tumour and patient characteristics as variables and is a prognostic or predictive model. We created a comprehensive nomogram in

chapter 6 which focuses on the prediction of a pathologically complete response after neoadjuvant chemoradiotherapy followed by surgery.
Randomized clinical trials can provide high level of evidence for medical decision-making, but it is unclear if the same results can be achieved in patients treated outside such trials. Also, the effectiveness and safety of a treatment for a patient who does not match the eligibility criteria of the trial participants is unsure.
In **chapter 7** the outcomes of patients with oesophageal cancer treated within and outside the randomized CROSS trial are being compared with the aim to validate CROSS in clinical practice.
In patients with extensive and bulky locoregional disease or patients with a strong suspicion of metastatic disease, induction chemotherapy (iCT) is indicated. The aim of induction chemotherapy is to induce tumour regression in order to make a complete resection of the primary tumour and metastatic sites possible [26].
In **chapter 8**, 124 patients are evaluated who underwent induction chemotherapy and underwent response monitoring with or without oesophagectomy. Chapter 9 evaluates, for the first time, the relation between pharmacokinetics of paclitaxel and response to treatment. These patients are either treated with induction chemotherapy, palliative chemotherapy or neoadjuvant chemoradiotherapy and clearance of paclitaxel is correlated to response. In patients treated with neoadjuvant chemoradiotherapy followed by an oesophagectomy, response was evaluated in the resection specimen (pathological response), whilst in patients treated with induction or palliative chemotherapy, response was evaluated by CT-scans (clinical response).

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Chapter two

New therapeutic strategies for squamous cell cancer and adenocarcinoma

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This paper presents commentaries on neoadjuvant treatment esophagectomy; the prognostic and predictive effects of single nucleotide polymorphisms (SNP) in the multimodality therapy of esophageal cancer; optimal preoperative treatment prior to surgery for esophageal cancer; a possible role for trastuzumab in treating esophageal adenocarcinoma or any esophageal dysplasia/intra-epithelial neoplasia; surgery after chemoradiation in resectable esophageal cancer; whether para-aortic lymph node dissection should be performed in esophagogastric junction (EGJ) tumors; and transhiatal esophagectomy in treatment of the esophageal cancer.

Keywords: HER2; transhiatal esophagectomy; transthoracic esophagectomy; SNPs; neoadjuvant chemoradiation; trimodality

Concise summaries

* Neoadjuvant chemoradiotherapy (CRT) treatment is favored by many worldwide with the aim to increase the percentage of radical resections and to reduce locoregional recurrences. There is no evidence that the modern schemes with low toxicity profiles increase postoperative morbidity or mortality; however long-term follow-up is needed to monitor late manifestations of (chemo)radiation-induced adverse effects.

* Genetic variations in drug action pathways are important in determining treatment response, and single nucleotide polymorphisms (SNPs) in these pathways seem to modify sensitivity or resistance to neoadjuvant treatment. They seem also to be effective as prognostic biomarkers and to have predictive value in the multimodality therapy of esophageal cancer.

* Pretherapy results in down-staging and pathological complete remission, which has been shown to be a surrogate for survival. It would thus appear that front-loading (induction) therapy is the most effective strategy to enhance surgical outcome for resectable cancers. Identification of treatment failure can spare patients from inactive therapy. Positron emission tomography (PET) responders to induction therapy will continue the same chemotherapy during subsequent combined CRT followed by surgery, whereas PET non-responders cross over to the other regimen during radiotherapy, to optimize pathologic response by changing chemotherapy.

* Clinical studies have demonstrated a survival benefit with both preoperative chemoradiation and chemotherapy. A major benefit of chemoradiation is to improve R0 resection and prevent local recurrence, but advances in systemic treatment are needed to improve outcome.

* Trastuzumab is a monoclonal antibody that targets the human epidermal growth factor receptor 2 (HER2) gene, also known as ErbB2, which encodes a transmembrane glycoprotein receptor, p185her2, with intracellular tyrosine kinase activity. Binding to the HER2 receptor induces antibody-dependent cellular cytotoxicity, inhibition of HER2-mediated signaling, and prevents cleavage of the extracellular domain of HER2. As data become available demonstrating the clinical benefit of the presence of HER2 in esophageal cancer, some have posed the question regarding targeting HER2 in early stage carcinogenesis such as treatment of HER2+ dysplasia.

* The available evidence reveals the need for accurate identification of patients that will benefit from surgery. However, no reliable test with a high negative predictive value (NPV) exists for differentiating responders from non-responders after chemo-radiation therapy. General reasons for the low accuracy of the available modalities include the difficulty of differentiating residual carcinoma from (CRT induced) inflammation or fibrosis, under detection of deep and spread residual foci, and non-standardization of techniques.

* Locally advanced tumors at the esophagogastric junction (EGJ) may present with para-aortic lymph node metastasis. Studies that assessed the pattern of lymphatic flow to the para-aortic nodes have identified several routes: directly from the left para-cardial nodes, from the nodes along the splenic artery, the celiac trunk, the superior mesenteric artery, and from the nodes along the posterior pancreatic head and common hepatic artery. The 5-year survival rate in patients with involved lymph nodes is low and, therefore, the benefit of routinely removing the para-aortic nodes remains questionable.

* Compared to transthoracic esophagectomy (TTE), transhiatal esophagectomy (THE) is associated with poor visualization of upper and middle thoracic esophageal tumors, potentially compromising the oncological integrity of the operation. However, the reported postoperative mortality after THE tends to be slightly lower than that of the transthoracic approach, but perioperative and oncological outcomes are not substantially influenced by the surgical approach to esophagectomy. Either procedure is associated with acceptable results in the hands of experienced surgeons.

1

Does neoadjuvant treatment increase peri-operative morbidity and/or mortality after esophagectomy?

Bas P.L. Wijnhoven and Eelke L.A. Toxopeus

INTRODUCTION

Multimodality treatment is considered the standard of care for nonmetastasized esophageal cancer. Neoadjuvant CRT is favored by many worldwide with the aim to increase the percentage of radical resections and to reduce locoregional recurrences. A recent meta-analysis on randomized controlled trials (RCTs) reported a survival benefit for CRT before surgery compared to surgery alone in patients with resectable esophageal carcinoma. The hazard ratio (HR) for all-cause mortality for neoadjuvant CRT was 0.78 (95% CI 0.70– 0.88; P < 0.0001); the HR for squamous cell carcinoma (SCC) only was 0.80 (0.68–0.93; P = 0.004) and for adenocarcinoma only was 0.75 (0.59–0.95; P = 0.02) [1]. The most recent published RCT, the CROSS trial from the Netherlands, endorses the use of neoadjuvant CRT plus surgery over surgery alone 2. While enthusiasts focus on the benefit of CRT, less attention is being paid to the adverse events. Little is known about the treatment-related toxicity and adverse effects in the longer term, such as radiation-induced cardiovascular and pulmonary complications.

RCTS: NEOADJUVANT CRT PLUS SURGERY VERSUS SURGERY ALONE

Fiorica *et al*. performed a meta-analysis of six RCTs and reported that the overall rate of postoperative adverse events was 39.4% in the CRT group and 34.4% in the surgery alone group. This difference was not significant. The most frequent adverse events were respiratory complications, heart failure, and anastomotic leak [3]. However, the risk of postoperative mortality—defined as death within 90 days—was higher in the CRT group in five RCTs. Combined analysis showed a significant effect of CRT on postoperative mortality with an odds ratio of 2.15 (95% CI 1.18–3.73). The included studies were published in the 1990s and patient accrual took place between 1983 and 1994. Hence, patient selection, CRT scheme (drugs, dose, fractionation, and sequence), and timing of surgery have changed significantly over time. Sensitivity analysis performed by excluding two trials with a fractionation dose of >2 Gy showed loss of significance [3].

The CROSS trial likely gives a more balanced picture of morbidity and mortality of CRT plus surgery [2]. Between March 2004 and December 2008, 366 patients were randomized to CRT followed by surgery (n = 178) and 188 patients to surgery alone. Patients treated with neoadjuvant CRT followed by surgery had a 34% lower risk of death during follow up (HR, 0.657).

Table 1 summarizes the postoperative events.

CRT was associated with a low frequency of highgrade toxic effects and could be given as an outpatient treatment. More importantly, the preoperative treatment did not result in higher postoperative morbidity or early mortality in this group, compared with the surgery group. Although morbidity was higher than expected and higher than reported in other studies, there was no plausible explanation for this finding, other than the fact that all postoperative events were meticulously recorded [2].

ADVERSE EFFECTS IN OBSERVATIONAL STUDIES

Although well-conducted RCTs may yield unbiased estimates of treatment effects, many RCTs fail to provide detailed adverse effects [4]. Explanations for this observation might be that the frequency of adverse events is low due to restrictive inclusion and exclusion criteria. Also, the number of included patients in RCTs is limited, and in frequent but harmful events may not occur within the follow-up period for the patients. As a consequence, systematic reviews on evidence from RCTs, though considered to be the highest level of evidence, often fail to provide accurate data on adverse effects. There is some evidence that observational studies on adverse effects are as valid as evidence from RCTs and should not be disregarded [5]. More empirical evidence indicates that there is no difference on average between the risk estimate of adverse effects of an intervention derived from meta-analyses of RCTs and meta-analyses of observational studies [6].

Reynolds et al. compared in-hospital postoperative morbidity and mortality in patients who underwent CRT and surgery with patients that received surgery alone [7]. Although the incidence of pneumonia and pleural effusion was similar between both groups, significantly more patients experienced sepsis, respiratory failure, and acute respiratory distress syndrome. Mortality was not significantly different between the two groups (7% and 4%, respectively). It is interesting to see that three out of six patients who died in the multimodal group had a complete pathological response and died without signs of sepsis. This report demonstrates that a detailed observation of patients is important in order to get insight into procedure-related complications. Although the anastomotic leak rate was similar between both groups in this study, van de Walle et al. reported on anastomotic complications after Ivor Lewis esophagectomy and neoadjuvant CRT [8]. They found that the complication rate is associated with a radiation dose to the gastric fundus. This further stresses the importance of a multidisciplinary approach in the treatment of patients with esophageal cancer. All physicians involved in the care should be well aware of the current protocols and outcome data on CRT and surgery for esophageal cancer.

SUMMARY AND FUTURE PERSPECTIVE

Recent evidence supports the use of multimodal treatment for esophageal carcinoma. There is no evidence that the modern CRT schemes with low toxicity profiles increase postoperative morbidity or mortality. However, careful long-term follow-up is needed to monitor late manifestations of (chemo)radiation-induced adverse effects. Although CRT is now considered a standard of treatment for esophageal cancer, one should keep in mind that patients enrolled in trials are selected and do not necessarily have similar characteristics to the population of esophageal cancer patients. The choice for neoadjuvant CRT compared to surgery alone should be discussed with the patient in the context of not only the possible benefit but also the harms of the treatment.

2

Prognostic and predictive effects of SNPs in the multimodality therapy of esophageal cancer

Daniel Vallbohmer and Wolfram T. Knoefel

INTRODUCTION

Multimodality therapy options have been introduced in the treatment of locally advanced esophageal cancer over the last years [9]. Nevertheless, results of current meta-analyses analyzing trials of different preoperative therapy protocols for patients with this malignant disease showed only moderate improvement of survival [1,10]. In contrast, the meta-analyses showed that patients with excellent response to neoadjuvant therapy seem to benefit from these preoperative regimens [1,10].

Consequently, for an individualized therapy approach, prognostic and predictive markers are highly needed in the multimodality treatment of locally advanced esophageal cancer [11].

Recent studies have demonstrated that genetic variations, especially in drug action pathways, are important in determining treatment response [12–15]. In particular, SNPs in these pathways seem to modify sensitivity or resistance to neoadjuvant treatment. In fact, current data suggest that SNPs can be used in the therapy of esophageal cancer for (1) prediction of an increased esophageal cancer risk, and as (2) prognostic biomarkers in the multimodality therapy of esophageal cancer and (3) predictive biomarkers in the multimodality therapy of esophageal cancer (Table 2) [12–14]. In the following short summary, one example for each of the three application fields where SNPs can be used is described and briefly discussed.

SNPS AND INCREASED ESOPHAGEAL CANCER RISK

So far, several molecular epidemiological studies have assessed the association between genetic polymorphisms and esophageal cancer risk [12]. Although the results are mostly encouraging, these studies are inadequate especially due to their retrospective design and low patient number. Interestingly, Yuan et al. performed a meta-analysis of 10 published case– control studies covering more than 6300 patients in order to evaluate the association between the xeroderma pigmentosum group D (XPD)Lys)751Gln polymorphism and esophageal cancer risk. XPD is involved in the nucleotide excision repair (NER) pathway, which plays a crucial role in the repair of DNA damages [12]. While the authors found no significant association between the XPDLys751Gln polymorphism and esophageal cancer risk in the whole study population (including esophageal squamous cell cancer and adenocarcinoma), they detected a significant association between the XPDLys751Gln polymorphism and adenocarcinoma [12].

SNPS AS PROGNOSTIC BIOMARKERS IN ESOPHAGEAL CANCER

Besides the evaluation of esophageal cancer risk, SNPs seem also to be effective as prognostic biomarkers in esophageal cancer. In fact, several studies have already shown a significant association between prognosis of esophageal cancer patients and SNPs. However, these studies are once again limited by their retrospective design and low patient number. For example, Bradbury et al. explored the prognostic significance of three vascular endothelial growth factor (VEGF; one of the most important angiogenetic factors) SNPs in 361 esophageal cancer patients [13]. They were able to demonstrate that the VEGF936C/T SNP was significantly associated with improved overall survival (OS) of the study patients.

SNPS AS PREDICTIVE BIOMARKERS IN ESOPHAGEAL CANCER

Finally, SNPs seem to have not only prognostic but also predictive value in the multimodality therapy of esophageal cancer. Yoon et al. assessed the X-ray repair cross-complementing protein 1 (XRCC1)Arg399Gln SNP in relation to the pathologic complete response rate of 81 patients with esophageal adenocarcinoma who received cisplatin based preoperative radiochemotherapy in a multicenter trial [14]. The authors detected the variant allele of the XRCC1 SNP (399Gln) in 52% of the study patients, whereas only 6% of the patients with this variant allele experienced a complete histopathologic response CR compared to 28% of the patients without this variant allele.

SUMMARY

Recent studies have identified different SNPs as predictive and prognostic biomarkers in esophageal cancer as well as for cancer risk assessment [12–14]. The current results are promising but still not implemented in clinical practice, as the available studies are mainly retrospective with low patient numbers. Therefore, large prospective studies are necessary to validate the potential role of SNPs in the therapy of esophageal cancer.

3

The optimal preoperative treatment prior to surgery for esophageal cancer is CRT

Mark J. Krasna

The advantages of neoadjuvant therapy include identifying those patients who are responders, improving tolerance of toxicity from therapy, downstaging tumor, possibly allowing for enhanced resectability and local control, and improving survival.

In the era of targeted therapy based on molecular markers for sensitivity/resistance and prognosis, obtaining tissue before therapy has begun and then tailoring therapy based on physiologic response (i.e., PET scan) or pathologic response is crucial.

A recent trial of concurrent erlotinib (150 mg/day) and radiotherapy (IMRT 60 Gy) for CRT-intolerant esophageal SCC patients reported the safety and efficacy of this approach. Patients were tested by immunohistochemistry (IHC) for epidermal growth factor receptor (EGFR); five of six patients were EGFR (3+). Eighteen patients with a median age of 71.5 years were studied. The median OS and progression-free survival (PFS) were 21.1 and 12 months, respectively. The 2-year OS, PFS, and locoregional relapse-free survival were 44.4%, 38.9%, and 66.7%, respectively. Recently, an RCT of neoadjuvant CRT followed by surgery versus surgery alone for locally advanced SCC of the esophagus was reported from China. Patients with IIB and III SCC of thoracic esophagus were given vinorelbine, cisplatin, X-ray therapy (XRT; 40 Gy), and three-hole esophagectomy. Of 123 patients, all finished the planned preoperative chemo-XRT and 49 underwent esophagectomy. The PCR rate was 29.6% and the R0 resection rate was higher (96.0% vs. 85.5%) after neoadjuvant therapy. Grade 3/4 toxicity included leukopenia (33 cases; 61.1%) and vomiting and esophagitis grade 1/2. There was no mortality; pulmonary infection was higher (8.2% vs. 1.4%, P = 0.094). The OS at 1 and 2 years between the arms was (85.6%/75.5% vs. 79.1%/66.1%, P = 0.207).

The rationale for the use of neoadjuvant therapy is that presurgical therapy does not significantly contribute to surgical mortality or morbidity. In addition, only 53% of patients completed their postsurgical therapy in previously reported trials. Pretherapy results in downstaging and pCR, which has been shown to be a surrogate for survival. Therefore, it would appear that front-loading (induction) therapy is the most effective strategy to enhance surgical outcome for resectable adenocarcinomas. Consistently demonstrated pathologic measures of improved OS after preoperative therapy and surgery include achievement of a pathologic CR, therapy treatment effect equaling or exceeding 90%, downstaging to node-negative status or earlier (T1–2) stage, and achievement of a negative margin (R0) resection [16–18].

Lin *et al.* evaluated 11 RCTs including 1,308 patients in a meta-analysis that demonstrated that neoadjuvant chemo-XRT improved OS. The OR was 1.28 (P = 0.05) for 1-year survival, 1.78 (P = 0.004) for 3 years; and 1.46 (P = .02) for 5-year survival. Postoperative mortality was increased in neoadjuvant CRT patients (P = 0.04), although postoperative complications were similar. Chemo-XRT lowered locoregional cancer recurrence (P = 0.04), although distant cancer recurrence was similar. Of note, SCC did not benefit from chemo-XRT; OR was 1 (P = 0.34) for 1-year survival, 1.34 (0.07) for 3-year survival and 1.41 (P = 0.06) for 5-year survival [16].

Although the MAGIC and FNLCC 94012/FFCD 9703 trials reported up to a 14% improvement in 5-year OS with perioperative ECF (epirubicin, cisplatin, and fluorouracil) or CF (cisplatin and fluorouracil), the EORTC trial 40954 and the U.S. INT 113 trial failed to improve OS with preoperative CF. The MRC OEO-2 trial employing preoperative CF indicated only a 6% improvement in 5-year OS and showed that improved OS was due to an improved rate of R0 resection with preoperative chemotherapy. The POET trial [19] of preoperative chemotherapy versus sequential chemotherapy followed by CRT showed that CRT achieved higher rates of pathologic CR (16% vs. 2%, P = 0.03), node-negative status (64% vs. 29%, P = 0.01); trends toward greater median survival (31 months vs. 21 months), 3-year OS (48% vs. 28%, P = 0.07), and improved 3-year local tumor control (77% vs. 59%, P = 0.06). There was no difference in rate of R0 resection (69–70%) between preoperative chemotherapy or CRT.

Identification of treatment failure as in the MUNICON trials can spare patients from inactive therapy. Non-responding patients have the potential to cross over to alternative therapies earlier on in treatment, as in the current CALGB trial using chemotherapy with either mFOLFOX-6 or weekly carboplatin and paclitaxel. PET responders to induction therapy will then continue the same chemotherapy during subsequent combined CRT followed by surgery, whereas PET non-responders cross over to the other regimen during radiotherapy to optimize pathologic response in non-responders by changing chemotherapy.

CALGB 9781 was the second RCT to show the benefit of trimodality (TRI). Tepper *et al.* [16] described 56 patients: 30 with trimodality and 26 with surgery alone. Grade 3 toxicities included hematological (54%) and GI (40%); there were 14 (SURG) and 17 (TRI) surgical complications in the two groups including two postsurgical deaths (SURG). Postoperative length of stay was 1.5 days (SURG) and 10 (TRI) days. There were 80% partial recovery (PR) and 40% pCR rates; median survival was 4.5 years (TRI) versus 1.8 years (SURG) (P = 0.02). Stratifications by N stage, staging, and histology demonstrated a P value of 0.005. The recent Dutch trial (CROSS) with 363 potentially resectable esophageal or EGJ cancer patients included 86 SCCs and 273 adenocarcinomas. Randomized to paclitaxel and carboplatin plus concurrent RT or surgery alone, the complete (R0) resection rate was higher with CRT (92% vs. 65%) and complete pathologic response was 33%. The median OS was significantly better with CRT (49 months vs. 26 months). Three year survival rate was 59% versus 48%. This regimen has become the standard for most locally advanced or nodal (stage II or III) disease [2]. A recent single institution review over 14 years included squamous (52) and adenocarcinoma (112) patients (164 total). The PCR rate was 41%; the OS was 46% (58% for PCR); locoregional control (LRC) was 79%. We found that squamous cancers fared better with regard to LRC (100%); and had higher PCR (54%). Those with M1a/residual disease had poor prognosis. Neoadjuvant CRT for esophageal cancer revealed possible stage-specific paradigms [16].

Patients with stage 0/1a are probably best treated with esophagectomy (MIE/THE). Those with stage 1b/2a can either receive surgery alone or neoadjuvant CRT, stage 2b/3a patients should be offered neoadjuvant CRT as the standard, and those with stage 3b/3c should receive either chemoradiation alone or neoadjuvant CRT.

4

The optimal preoperative treatment prior to surgery is chemotherapy?

Kimberly Perez

Preoperative chemotherapy is a controversial topic in the treatment of locally advanced esophageal carcinoma. The 5-year survival of all patients with esophageal cancer has improved modestly over the last 30 years, from 5% to 19% [20]. The slight improvement may be partially attributed to evolution over the last 15 years in the management of locally advanced disease. Over the last two decades, the role of systemic chemotherapy in multimodality therapy has been evaluated. Despite the addition of systemic chemotherapy, less than a third are cured by trimodality therapy, and 65% demonstrate distant recurrence [21]. The role of prognostic characteristics and targeted therapies in clinical management is currently under investigation. Although preoperative chemoradiation provides significant improvement in OS, the modest survival rates warrant evaluation of these modalities.

Multiple randomized trials have evaluated the role of chemotherapy in the preoperative setting. Three trials, the MRC, MAGIC, and FNLCC/FFCD trials, further supported by a 2011 meta-analysis of nine trials, demonstrated a survival benefit of the addition of chemotherapy to surgery, compared to surgical resection alone. The average OS advantage was 35% compared to 20% for surgery alone. In the MAGIC trial, local recurrence was confirmed in 14% of the peri-operative treatment group compared to 20% in the surgery-alone group; distant metastases were noted in 24% and 36% patients, perioperative and surgery alone, respectively [22]. In the MRC trial, the rates of local recurrence and distant metastases were similar in both groups, 8% and 12%, respectively [23]. In the FNLCC/FFCD trial, distant sites of recurrence were lower at 30%, compared to 38%; locoregional recurrence was similar at 12% and 8% in preoperative and surgical groups, respectively [24].

The addition of preoperative CRT has resulted in improved resection rates and LRC. The CROSS trial compared preoperative CRT to surgery alone. The complete (R0) resection was significantly

higher for CRT (92%) compared to surgery alone (69%). At a median 45.4 months of follow-up, 85% of patients who received chemoradiation died due to cancer recurrence; this is in contrast to 94% who died in the surgery-alone group. The OS was significantly better in the chemoradiation group, with a HR of 0.657 (95% CI, 0.495–0.871; P = 0.003) [2]. Stahl et al. attempted to compare preoperative chemotherapy with preoperative chemoradiation. Despite not meeting accrual goals in this study, the rate of R0 surgical resection was not significantly different, 69.5% and 71.5% for chemotherapy and chemoradiation, respectively. The rate of local tumor progression at 3 years was 59% and 76.5% (P = 0.06), chemotherapy and chemoradiation, respectively. The median survival was 21.1 months after preoperative chemotherapy and 33.1 months after preoperative chemoradiation [25]. With other trials supporting improved survival with preoperative concurrent chemoradiation [2] combined modality for potentially resectable stage II or III localized cancer of the thoracic esophagus is preferred to chemotherapy alone.

In summary, clinical studies have demonstrated a survival benefit with both preoperative chemoradiation and chemotherapy. A major benefit of chemoradiation is to improve R0 resection and prevent local recurrence. Systemic metastases are the main source of recurrence and ultimate death after trimodality treatment of esophageal cancer. Advances in systemic treatment are needed to improve outcomes. Trials are currently underway using PET scan to assess the effectiveness of systemic therapy. The effects of these modalities will likely revolutionize the next 15 years of esophageal cancer therapy.

5

Trastuzumab: is there a role for distal esophageal adenocarcinoma, or any potential for treatment for esophageal dysplasia/intraepithelial neoplasia?

Kimberly Perez

The HER2 gene, also known as ErbB2, encodes a transmembrane glycoprotein receptor, p185her2, with intracellular tyrosine kinase activity. Recent studies demonstrate rates of HER2 overexpression around 20% in esophageal carcinoma [26–29], which is comparable to expression demonstrated in primary invasive breast adenocarcinomas of 18–20%. The clinical importance of perioperative trastuzumab to prevent recurrence in HER2+ esophageal adenocarcinoma is being evaluated in phase III trials. The potential role of targeting HER2 for HER2+ esophageal dysplasia is under investigation.

Trastuzumab is a monoclonal antibody that targets HER2. Binding to HER2 receptor induces antibody-dependent cellular cytotoxicity and inhibition of HER2-mediated signaling, and prevents cleavage of the extracellular domain of HER2. Four trials of women with resected HER2+ breast cancer demonstrated a 37–49% increase in survival with trastuzumab [30–32]. As a result, trastuzumab is currently FDA approved in patients with HER2+ metastatic breast cancer.

The ToGA trial was a phase III, randomized controlled study in which 3807 metastatic gastric cancer patients (32% GEJ) were screened for HER2 expression; 810 (22.1%) patients were positive; 584 of these patients were randomized to a fluoropyrimidine–cisplatin based regimen with or without trastuzumab. The OS and tumor response rates were improved with the trastuzumab combination; OS was 13.8 months and 11.1 months and overall response rates were 47.5% and 34.5%, with and without trastuzumab, respectively [27].

Current standard treatment of locally advanced esophageal cancer includes trimodality therapy with chemoradiation followed by surgical resection. After the results of the ToGA trial, the next logical step was to determine if trastuzumab increased cure rates in early stage esophageal cancer patients. RTOG 1010 opened in January 2011 as a phase III randomized controlled study of trastuzumab and chemoradiation for EAC. Patients with 3+ IHC expression or positive fluorescence in situ hybridization (FISH) are being enrolled and randomized to a treatment arm with or without trastuzumab. Approximately 160 patients will be enrolled to determine if the addition of trastuzumab increases PFS from 15 to 27 months [33].

As data become available demonstrating the clinical benefit of the presence of HER2 in esophageal cancer, some have posed questions regarding targeting HER2 in early stage carcinogenesis such as treatment of HER2+ dysplasia. BE is a premalignant lesion that precedes adenocarcinoma through multiple steps of dysplastic lesions. HER2 overexpression has been demonstrated in dysplastic tissue at a rate of 35–50% [34–37]. Villanacci et al. treated two patients diagnosed with BE with trastuzumab. There was no change in endoscopic and histologic patterns; however there was downregulation of HER2 with increased apoptosis [38].

Treatment of gastric cancer with trastuzumab has opened the door to further evaluation of HER2 in esophageal carcinoma and dysplasia. Data are not mature; however, preliminary data support the potential clinical role of targeting HER2.

6

Surgery after chemoradiation in resectable esophageal cancer

Peter S.N. van Rossum and Jelle P. Ruurda and Richard van Hillegersberg

Preoperative CRT has recently become the standard of care for patients with potentially curable esophageal cancer, improving the percentage of complete resections (R0) from 69% to 92% and the 5-year OS rate from 34% to 47% [2]. The current gold standard CRT regimen as proposed by the Dutch CROSS trial consists of weekly administration of carboplatin and paclitaxel for 5 weeks and concurrent radiotherapy (41.4 Gy in 23 fractions) followed by surgery. This regimen is associated with low perioperative mortality (4%), a high proportion of pathological complete responders (up to 49% in squamouscell carcinoma) and acceptable adverse-event rates 2. Earlier meta-analyses further support this standard by showing a 7–13% survival benefit for preoperative CRT compared to surgery alone [1–10].

Recent data from rectal cancer studies suggested that surgery might be omitted in patients with a complete response to neoadjuvant treatment [39]. The current evidence supporting this "wait-and-see" approach in esophageal cancer patients, however, is based on only two RCTs [19,40]. The results of these trials are difficult to interpret because of complex trial designs, relatively old data with long accrual times (over 7 years), old treatment regimens, and inferior results compared with the current gold standard.2 Namely, the treatment-related mortality is high (9–13% vs. 4%) and survival rates low (34–40% vs. 67% after 2 years) [19,40].

In the study by Bedenne *et al.* [40] 444 patients received two cycles of fluorouracil and cisplatin and either conventional (46 Gy) or split-course (30 Gy) concomitant radiotherapy. Out of 259 clinically responding patients with mainly SCCs (89%), 129 were randomly assigned to surgery (arm A), and 130 were assigned to another three cycles of CRT (arm B). The conclusion that there was no benefit for the addition of surgery after CRT was mainly based on the nonsignificant difference in the 2-year survival rate between arm A and arm B (34% vs. 40%, P = 0.44). However, this result was negatively influenced by the high rate of peri-operative/in-hospital mortality in the surgery arm. Nevertheless, in the surgery arm, the local tumor recurrence was significantly lower (34% vs. 43%, P = 0.001), stents for stenoses were considerably less required (5% vs. 32%, P = 0.001), a palliative procedure against dysphagia was less often performed (24% vs. 46%, P < 0.001), and the quality of life was equal (P = 0.26).

Stahl *et al.* [19] randomly allocated 172 patients with locally advanced SCC to either induction chemotherapy with three courses of bolus fluorouracil, leucovorin, etoposide, and cisplatin, followed by CRT (cisplatin and etoposide plus 40 Gy) and surgery (arm A), or the same induction chemotherapy followed by CRT (cisplatin and etoposide plus at least 65 Gy) without surgery (arm B). The OS after 2 years was equivalent in the two groups (40% vs. 35%, log-rank test for equivalence, P = 0.007), but the survival curves for OS seemed to spread after 3 years (without

reaching statistical significance). Moreover, the 2-year PFS was considerably better in the surgery group (64% vs. 41%, P = 0.003). The importance of achieving high PFS (e.g., local control) is demonstrated by a review of nine series including a total of 105 patients undergoing salvage esophagectomy for local recurrence after definitive CRT, which is commonly performed for this indication [41]. An overall anastomotic leakage rate of 17%, a treatment-related mortality rate of 11%, and 5-year survival rates of 25–35% were reported, implying unfavorable outcomes when compared to outcomes after subsequent surgery after CRT [2,41].

Thus, rather than questioning the need for surgery in esophageal cancer, the available evidence reveals the need for accurate identification of patients that will or will not benefit from surgery. However, no reliable test exists for differentiating responders from non-responders after CRT. Ideally, a test with a high NPV for the detection of tumor residue is warranted in order to clinically identify complete responders who may avoid surgery. Reported NPVs of commonly used modalities for response evaluation such as esophagraphy, endoscopy (with or without biopsy), and endoscopic ultrasound (EUS), however, disappointingly range from 0% to 67%, 31% to 59%, and 6% to 47%, respectively. 18Fluorodeoxyglucose PET (FDG-PET) seemed more promising regarding the purpose of accurately evaluating the response to CRT. However, two recent systematic reviews revealed pooled sensitivities of only 67–70% and pooled specificities of 68–70%, concluding that routine implementation in clinical practice for this purpose is currently unjustified [42,43]. General reasons for the low accuracy of the available modalities include the difficulty of differentiating residual carcinoma from (CRT-induced) inflammation or fibrosis, underdetection of deep and spread residual foci, and non-standardization of techniques.

The current available evidence indicates that the key to success in patients with resectable esophageal cancer is a multimodality treatment regimen. CRT followed by surgery is the gold standard, providing the best chance for local tumor control and longterm survival with low toxicity. First, studies are needed to develop and optimize strategies for accurate response evaluation before patient subgroups might safely be omitted from surgery in the future, and a higher percentage of complete responders has to be achieved by optimization of radiotherapy in order to justify a nonsurgical approach.

7

Should para-aortic lymph node dissection be performed in EGJ tumors?

Marc Schiesser and Paul Schneider

EGJ tumors exhibit a rising incidence. Lymph node metastasis is one of the most important prognostic factors for gastric and EGJ tumor [44,45]. The extent of lymphadenectomy (LAD) has been a matter of discussion for several decades. Centers from Japan have particularly been advocating extensive LAD. The LAD of the lower mediastinum and a D2 LAD have been proposed as an international standard for EGJ tumors, resulting in an improved prognosis for these kinds of tumors [44,46,47].

However, locally advanced tumors may even present with para-aortic lymph node metastasis. The value of routine or selected para-aortic LAD for EGJ tumors remains unclear. Studies that assessed the pattern of lymphatic flow to the para-aortic nodes have identified several routes: (1) directly from the left paracardial nodes, (2) from the nodes along the splenic artery, (3) from the nodes around the celiac trunk, (4) from the nodes along the superior mesenteric artery, and (5) from the nodes along the posterior pancreatic head and common hepatic artery [48]. Risk factors for para-aortic lymph node metastasis are macroscopic N stage, tumor size, and the involvement of the lymph node station Nr. 7 along the left gastric artery, according to Nomura *et al* [49].

In gastric cancer, a recent randomized clinical trial with 523 patients assessing the value of routine para-aortic LAD did not show a benefit regarding overall and recurrence free survival [50]. However, the incidence of para-aortic lymph node metastasis in this trial was only 8–5%, and only 20% of the tumors were proximal gastric cancers. Therefore, no clear comparison can be drawn from this patient population compared to EGJ tumors. Interestingly, the complication rate was not different and the procedure appeared to be safe in expert hands. Studies that assess esophagogastric tumors are rare. Yamashita et al. assessed the optimal extent of LAD in 225 patients with Siewert type II tumors [51]. They dissected the para-aortic lymph node nodal station 16a2 in 73 patients and the nodal station 16b in 38 patients. The lymph node metastasis rate was 11% for 16a2 and 18.4% for 16b. The 5-year survival rate in patients with involved lymph nodes was 12.5% for 16a2 and 0% for 16b. Similar results were shown by a recent study [52]. Therefore, the benefit of routinely removing the para-aortic nodes remains questionable due to the low incidence and the low survival rate (Table 3). Further studies should be advocated in this patient population. We believe that para-aortic lymph node resection in selected patients might be a valuable alternative to routine para-aortic LAD. Novel imaging strategies to identify patients with involved lymph nodes such as FDG-PET/CT will help to establish such tailored surgical approaches [53]. Frozen sections from the lymph node station 7 next to the left gastric

artery might be another alternative strategy to identify patients at risk for para-aortic lymph node involvement.

In conclusion, para-aortic LAD is safe in expert hands and selected patients might benefit from para aortic LAD. However, the benefit of para-aortic LAD remains questionable and should not be performed on a routine basis.

8

Is there a place for transhiatal esophagectomy in treatment of the esophageal cancer?

Valter Nilton Felix

There are two main histopathological subtypes of esophageal cancer: SCC and adenocarcinoma. SCC is the most common sub type in several regions of the world. On the other hand, adenocarcinoma is commonly associated with Barrett's metaplasia, GERD, and obesity and frequently involves the GEJ and proximal stomach.

Multiple approaches have been described for esophagectomy, and they can be thematically categorized under two major headings: transthoracic or transhiatal. The theoretical advantage of the transthoracic approach is a more thorough oncological operation as a result of direct visualization and exposure of the thoracic esophagus, which allows a wider radial margin around the tumor and more extensive lymph node dissection. However, the combined effects of an abdominal and thoracic approach might compromise cardiorespiratory function, especially in patients with coexisting lung or heart disease. The perioperative mortality of TTE in experienced centers ranges from 9% to as low as 1.4%. Five-year survival in approximately 25% of patients who undergo transthoracic esophageal resection has been reported. However, these reports include heterogeneous populations of patients with esophageal cancer that underwent a variety of surgical approaches, the use of adjuvant treatment in some but not all patients, and combined histology.

The transhiatal approach is performed through midline laparotomy or laparoscopy and left cervical incision. The abdominal portion of the THE includes mobilization of the stomach, pyloromyotomy, and placement of a feeding jejunostomy. After access to the mediastinum and dissection under direct vision of the distal and middle third of the esophagus, a left cervical incision along the anterior border of the sternocleidomastoid muscle provides exposure to the cervical

esophagus. Circumferential dissection of the cervical esophagus is carried down to below the thoracic inlet, and blunt dissection is continued into the superior mediastinum to mobilize the upper thoracic esophagus. The remainder of the dissection at the level of and superior to the carina is completed by blunt dissection through the esophageal hiatus. The cervical esophagus is then divided, the stomach and attached intrathoracic esophagus are delivered through the abdominal wound, and a gastric conduit is fashioned using a linear stapling device. The gastric tube is delivered through the retrosternal route to the cervical wound, where a cervical esophagogastric anastomosis is performed. The stomach is considered by most surgeons as the ideal replacement for the resected esophagus, although a segment of colon or a free flap of small bowel can be used as alternative conduits.

The postulated advantages of the transhiatal approach to esophagectomy are minimized pain, subsequent postoperative pulmonary complications, and a shorter duration of operation, which potentially results in decreased morbidity and mortality. Compared to TTE, THE is associated with poor visualization of upper and middle thoracic esophageal tumors, potentially compromising the oncological integrity of the operation. However, the reported postoperative mortality after THE tends to be slightly lower than that of the transthoracic approach, between 1% and 7.5% [54], and 5-year survival rate is approximately 25%, which is not substantially different from that accomplished after the transthoracic approach.

A randomized trial, published by Hulscher *et al.* [55] provided level I evidence regarding this controversial issue. Two hundred and twenty patients were assigned to either transhiatal or TTE with cervical anastomosis. The TTE procedure included en bloc resection of the thoracic duct, azygos vein, ipsilateral pleura, and all peri-esophageal tissue in the mediastinum, including a formal LAD. THE had a shorter operative duration than TTE (3.5 h vs. 6 h), with lower blood loss (1 L vs. 1.9 L). Perioperative pulmonary complication rate was also lower in the transhiatal group (57% vs. 27%). Duration of mechanical ventilation, ICU stay, and hospital stay were all shorter in the transhiatal group. However, there was no significant difference in hospital mortality (TTE: 4%; THE: 2%). Although initially a trend toward a survival benefit was seen with the transthoracic approach, after longer follow-up, no difference in 5-year OS was found (TTE: 36%; THE: 34%).

A large population-based study was published recently [56], in which a lower operative mortality was found after THE (6.7% vs. 13.1%) and no significant 5-year survival difference was found. These data suggest that perioperative and oncological outcomes are not substantially influenced by the surgical approach to esophagectomy, and that either procedure is associated with acceptable results in the hands of experienced surgeons. Ideally, surgeons and hospitals treating patients with esophageal carcinoma should have expertise in both techniques. Some patients might even benefit from an individualized approach. For an older or higher risk surgical patient, for whom perioperative recovery is an even greater concern than usual, a transhiatal approach could confer an advantage. In a fit patient with evidence of a limited number of involved lymph nodes, there is some evidence (although not level I evidence) that suggests a benefit in survival with the transthoracic approach. Still, available literature suggests that the experience of the surgeon and hospital is likely to be a more important factor than the type of approach selected.

A meta-analysis [57] has reported that preoperative CRT improved 3-year survival by 13% over surgery alone, with similar improvement identified in patients with either SCC or adenocarcinoma histology. Although the role of surgery has been questioned, especially for SCC, it can be rea-

sonably concluded that esophageal resection remains an important, if not the most important, therapeutic component of a combined modality approach to esophageal cancer. Surgeons interested in this lethal disease should direct their efforts to more accurate identification of those patients that will likely benefit from different single or combination treatment modalities, and tailor their therapeutic interventions accordingly. Perhaps actual radical LAD has not sufficient extension to give real potency to surgery to increase the survival rates if the thoracic approach is adopted and, for example, a bilateral thoracoscopic procedure could provide superior oncological outcomes.

Considering actual surgical proceedings, neither approach has consistently proven to be superior to the other one. Moreover, the available literature suggests that the experience of the surgeon and hospital in the surgical management of esophageal cancer is an important factor for operative morbidity and mortality rates, which could supersede the type of approach selected. Oncological outcomes appear to be similar and not so good after both procedures.

Tables

Table 1: Adverse events after CRT plus surgery and surgery alone

Postoperative events no. of patients/total no. (%)	CRT plus surgery (*n* = 171)	Surgery alone (*n* = 186)
Pulmonary complications	78/168 (46)	82/186 (44)
Cardiac complications	36/168 (21)	31/186 (17)
Chylothorax	17/168 (10)	11/186 (6)
Mediastinitis	5/168 (3)	12/186 (6)
Anastomotic leakage	36/161 (22)	48/161 (3)
In hospital death	6/168 (4)	8/186 (4)
Death after 30 days	4/168 (2)	5/186 (3)

Table 2: Application fields of SNPs in the multimodality therapy of esophageal cancer

Application field	Example	Reference
Esophageal cancer risk assessment	Significant association between *XPD*^{Lys751Gln} SNP and esophageal adenocarcinoma	12
Prognostic biomarker	Significant association between *VEGF*$^{936C/T}$ SNP and improved overall survival	13
Predictive biomarker	Association of *XRCC1*^{Arg399Gln} SNP and histopathologic response in esophageal cancer patients	14

Table 3: Incidence of para-aortic lymph node metastases

Author	Tumor type	*n*	Incidence
Nomura *et al.*[45] (2007)	Gastric cancer	260	8.3 %
Sasako *et al.*[46] (2008)	Gastric cancer	523	8.5 %
Yamashita *et al.*[47] (2011)	Siewert type II	225	11-18.4 %

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PART I

Potential Biomarkers

Chapter three

MET protein expression in esophageal adenocarcinoma: a mislead for targeted therapy?

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Abstract

AIM

The aim of this study was to assess MET expression and its prognostic significance in a large cohort of patients with EAC.

MATERIALS AND METHODS

A tissue micro array was constructed from 348 samples of patients with esophageal or gastro-esophageal junction adenocarcinoma (EAC) who underwent esophagectomy between 1995 and 2007. Specificity of MET antibodies D1C2, Sp44 and 3D4 were tested by Western blotting and immunocytochemistry.
Tumor cores were stained with a specific C-terminus MET antibody D1C2, and expression was assessed by scoring membranous and/or cytoplasm expression. Survival and univariable and multivariable Cox regression analyses were performed to identify its prognostic role.

RESULTS

D1C2 was the most specific antibody for MET. Tumor cores were sufficient from 262 patients (75.3%) and were included in final analysis. A positive correlation was found between MET expression and five-year overall survival (35% versus 20% survival in high versus low cytoplasm expression, p=0.083). Multivariable analysis revealed pT3 stage (HR 2.43, 95% CI: 1.11 – 5.31), increasing pN stage (HR 1.73, 95% CI: 1.06 – 2.81; HR 2.27, 95% CI 1.41 – 3.64 and HR 3.48, 95% CI 2.11 – 5.73) and radicality of the resection (HR 0.66, 95% CI: 0.47 – 0.93) as independent predictors of survival, without an impact for MET expression.

CONCLUSION

High cytoplasm MET expression in EAC is associated with a favorable five-year survival. This observation puts the previously reported negative prognostic impact

Introduction

Esophageal cancer is an aggressive disease, with rising incidences in the United States and Western Europe [1,2]. It is often diagnosed at an advanced stage. Hence less than half of the patients are eligible for curative treatment, for which surgery remains the cornerstone. Advances in surgical techniques and the introduction of neoadjuvant therapy have improved both short and long-term outcome, for which TNM-stage is the strongest prognostic factor [3–7]. However, the accuracy of clinical tumor staging is still moderate. Molecular markers may improve prognostication but can also serve as potential targets for therapy.

Receptor tyrosine kinase (RTK) receptors, such as the hepatocyte growth factor (HGF) receptor MET are attractive candidates [8]. MET is a transmembrane RTK receptor on epithelial cells with oncogenic properties. When MET is activated by its natural ligand HGF, phosphorylation of the receptor leads to activation of downstream intracellular signaling pathways involved in cell proliferation, survival, growth, motility, invasiveness, degradation of the extracellular matrix, mesenchymal transformation and meddling of cell-cell junctions [9,10]. Overexpression of MET due to upregulation by HGF, MET gene amplification or mutations is seen in several neoplasms [11,12]. In esophagogastric cancers, MET amplification is rare and observed in only 2% of cases 13. However, it is reported that an increase in MET gene copy number is associated with higher tumor grade and stage and with decreased overall survival [13,14]. Hence selective MET receptor inhibition could be a promising development in anticancer treatment. In esophageal adenocarcinoma (EAC) high expression of MET has been associated with poor overall survival [15–17]. In contrast, high MET expression in gastric cancer has been associated with a more favorable prognosis [18].

Targeting MET in patients with EAC could be of potentially clinical relevance. In a recently published review, an overview of clinical data of agents specifically targeting MET in esophagogastric cancer was presented [19]. A randomized phase 3 trial including patients with both locally advanced or metastatic MET-positive gastric, lower esophageal, or gastroesophageal junction adenocarcinoma, however, showed that ligand-blocking inhibition of the MET pathway with rilotumumab added to standard chemotherapy did not improve clinical outcomes in these patients (RILOMET-1 trial, [20]). Another phase 3 clinical trial with rilotumumab in this population is still ongoing (RILOMET-2 trial, [21]). Furthermore, onartuzumab, another MET inhibitor was used is the randomized controlled trial of patients with metastasized gastroesophageal carcinoma. Patients were randomly assigned to either FOLFOX with or without onartuzumab if tumors were Sp44 MET positive. There was no difference in survival of patients treated with addition of onartuzumab [22].
Of significant impact is that different antibodies are used to evaluate MET immunoreactivity in esophageal and gastroesophageal junction adenocarcinomas. Only little attention has been paid to the specificity and sensitivity of these antibodies [15,17,18,23,24].Therefore, the validity and reproducibility of MET antibodies should first be considered before the prognostic role of MET expression in EAC can be determined. The aim of this study was to assess MET expression in EAC using a proven specific monoclonal MET antibody and to correlate MET expression with prognosis and to hitherto critically (re)consider the potential role of MET inhibition in EAC.

Materials and methods

PATIENT SELECTION

Patients with histologically proven EAC who underwent an esophagectomy in the Erasmus MC, University Medical Center Rotterdam between 1995 and 2007 were identified from an institutional database. A small proportion of these patients underwent neoadjuvant chemotherapy, radiotherapy or a combination of both. Ethical approval was obtained by the medical ethical committee of the Erasmus MC, University Medical Center Rotterdam (MEC-12-469).

STAGING

All resection specimens were staged according to the 7th UICC-AJCC TNM staging manual [25]. A radical resection (R0) was defined as no tumor cells within 1 mm of the circumferential, proximal or distal resection margins.

TISSUES

Samples of the tumor, lymph nodes and resection margins were obtained from the resection specimens and fixed in formalin and embedded in paraffin. Formalin fixed, paraffin embedded (FFPE) tumor blocks were retrieved from the department of pathology and an H&E slide was made and reviewed by an experienced gastrointestinal pathologist (FJWtK). Tumor tissue was identified and marked and with a 16-gauge needle four to six 0.6 mm cores from different areas of the primary tumor were taken from the FFPE blocks (donor block) were manually bored and arrayed in a multi-tissue straw in a recognizable pattern (Figure 1A). Also, known organ system controls for immunohistochemistry were included on the Tissue Micro Array (TMA) [26].

MET IMMUNOHISTOCHEMISTRY

Sections of 4 µm thickness were cut from the TMA, mounted on glass slides and deparaffinized with three xylene rinses (5 min) and rehydrated in ethanol (3 x 100%, 2 x 96%, 1 x 70% and 1 x 50%). After washing with H_2O, endogenous peroxidase activity was inactivated by incubating the sections in 3% H_2O_2 for 10 min. After washing with H_2O, antigen retrieval was carried out by heating the sections under high pressure – up to 1.2 bar – in Tris-EGTA buffer (0.01 M Tris, 0.001 M EGTA, pH 9.0). Endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (SP-2001; Vector Laboratories, Inc.; Burlingame, CA 94010, USA) according to the manufacturer's protocol. After washing with 1X PBS, the sections were incubated with 0.22% bovine serum albumin (BSA) solution (A7034; Sigma-Aldrich®; Zwijndrecht, The Netherlands) in 1X PBS for 7 min to reduce non-specific background expression. Subsequently, the sections were incubated overnight at 4 °C with the monoclonal rabbit anti-human MET antibody (1:100; D1C2; Cell Signaling Technology® Europe B.V., Leiden, The Netherlands). After washing with 0.1% Tween-20 in 1X PBS, the sections were incubated for 30 min with 1:150 biotinylated polyclonal swine anti-rabbit secondary antibody (E0431; Dako; Heverlee, Belgium). After washing with 0.1% Tween-20 in 1X PBS, sections were incubated for 20 min with an avidin-biotinylated horseradish peroxidase complex that was prepared according to the manufacturer's protocol (PK-6100; Vector Laboratories, Inc.). Finally, after washing with 0.1% Tween-20 in 1X PBS and 1X PBS, horseradish perox-

idase activity was visualized by incubating the sections for 2x 5 min with 3, 3'-diaminobenzidine prepared according to the manufacturer's protocol (K3468; Dako). All antibodies were diluted in 0.22% BSA solution in 1X PBS. The sections were counterstained with hematoxylin before mounting them with a coverslip.

ASSESSMENT OF MET EXPRESSION

Assessment of MET expression was performed by a gastrointestinal pathologist (FJWtK). Staining was used as parameter for MET expression. Presence of tumor, membranous and cytoplasm staining of MET in cancer cells was semi quantitatively scored using a four-step scale from 0 to 3: 0: no staining or equal to background; 1, weak staining; 2, moderate staining and 3, strong staining (Figure 1B). Only when more than 50% of the cores per case were present and eligible, the patient was included for statistical analysis. All tumor cells within core were taken into account. The average of the staining intensity of all cores was calculated and rounded to whole numbers (again 0, 1, 2 and 3) for membranous and cytoplasm staining respectively. Low MET expression represented a combination of 0 and 1 scored staining, and high MET expression represented 2 and 3 staining together.

MET EXPRESSION IN ESOPHAGEAL CANCER CELL LINES

To test the D1C2 antibody in esophageal cancer cells, a TMA of fourteen well characterized EAC cell lines and eighteen squamous cell carcinoma cell lines were used (annotation in supplementary data). The TMA was incubated with the antibody against MET (1:400) according to earlier described protocol, with the only modification that the antigen retrieval occurred under 0.9 bar. Immunoreactivity was evaluated in each cell line using a four-step scale from 0 to 3 for membranous and cytoplasmatic expression.

WESTERN BLOT ANALYSIS OF ANTIBODY SPECIFICITY

A selection of commercially available C-terminus MET antibodies (SP44, D1C2 and 3D4, see below) were used to assess the specificity of MET expression. Pellets from cell lines with known MET expression (MET positive HT-29 and MET negative LNCaP), nine EAC cell lines (ESO 26, FLO-1, P4CE, ESO 51, I2425, OE19, M5.1, OE33 and KYAE) and tissue biopsies (three fresh diagnostic adenocarcinoma biopsies) were homogenized in 1x Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) and heated at 95 °C for 3 minutes. Proteins were separated on 8% SDS-PAGE gels and transferred to Immobilon-FL Transfer membranous (Millipore, Bellerica, USA). The membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, USA) for 1 hour at room temperature and incubated overnight at 4°C with anti-MET antibodies 3D4 (mouse monoclonal, 2 µg/ml, 1:250, Life Technologies), SP44 (rabbit polyclonal 1:1000, Spring Bioscience) or D1C2 (rabbit monoclonal, 1:1000, Cell Signaling Technology, Inc.; Danvers, MA 01923, USA). For loading control mouse anti-beta-Actin monoclonal antibody (1:5000, Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit anti-beta-Tubulin (1:10,000, Abcam, Cambridge, UK) were used. Incubation with secondary antibodies (Goat-anti-mouse IgG IRDye 680CW and Goat-anti-rabbit IgG 800CW both 1:5000; LI-COR Biosciences, Lincoln, United States) was at room temperature, for 1 hour. Blots were scanned using an Odyssey Infrared Imager (LI-COR Biosciences) and the results were analyzed using Odyssey software.

RNA-MET EXPRESSION IN EAC CELL LINES

EAC cell lines (ESO 26, FLO-1, P4CE, ESO 51, I2425, OE19, M5.1, OE33 and KYAE) were analyzed for MET expression at the mRNA level. Total RNA was extracted, reverse transcription was performed with 500 ng RNA (PrimeScript™ 1st strand cDNA Synthesis Kit, Takara) according to manufacturer's instruction and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SensiMix SYBR and Fluorescein Kit (Bioline, London, United Kingdom) and BioRad PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction. Primers used for MET detection were designed (forward, TGTCCCGAGAATGGTCATAA; reverse, AGGGAAGGAGTGGTACAACA) and synthesized by Sigma-Aldrich (Sigma-Aldrich Chemie B.V. Zwijndrecht, Netherlands) [27]. Gene expression level of MET was normalized using the ΔΔCT method and HPRT as reference gene (forward, ACCAGTCAACAGGGGACATAA; reverse, CTTCGTGGGGTCCTTTTCACC).

STATISTICAL ANALYSIS

Associations between patient and tumor characteristics and MET immunoreactivity were evaluated using the Pearson's Chi-square test. For survival analysis, the cohort of patients was stratified in two groups according to MET expression by taking the median of membranous and cytoplasm expression (below or on the median (weak expression) and above the median (strong expression)). When membranous and cytoplasm expression were combined, median numbers were recoded and divided into three categories; weak, moderate and strong expression. Sensitivity analysis was done when patients receiving neoadjuvant chemo- or chemoradiotherapy were excluded. Overall 5-year survival rates were estimated according to the Kaplan-Meier method and compared between groups with the log-rank test. Overall survival was calculated between date of esophagectomy and date of death, irrespective of the cause, or till last day of evaluation. , e.g. November 21st of 2013, with for every patient more than 5-years of follow up. To identify prognostic factors affecting survival, a univariable analysis was performed. Relevant clinicopathological characteristics were taken into account and variables with a value of $p < 0.1$ together with clinically relevant variables were included in a multivariable Cox regression model. Statistical significance was set at the 5% level (two-sided). Data are expressed as hazard ratios +/- 95% confidence intervals (CI). Statistical calculations and analysis were performed with the use of SPSS software, version 25.0 (SPSS, IBM, New York, NY, USA).

Results

MET expression in tumor samples

Of the 348 available tissue samples, the quality of the tissue cores was insufficient in 86 samples due to absence of sufficient numbers of cores or absence of tumor cells. Therefore, a total of 262 tissue samples (75.3%) were included in the analysis. Fourty-five patients had undergone neoadjuvant therapy (17%). Patient and tumor characteristics are summarized in Table 1. High cytoplasm expression of MET was seen in 65% of the tumors, while high membranous expression was present in 28% of the patients. MET cytoplasm expression was more prominent than membranous expression and high membranous expression of MET was always accompanied with cytoplasm expression (Table 1).

Survival analysis and prognostic factors

The median overall survival of all patients was 21 months (IQR 8 – 62). The median overall survival of patients after esophagectomy with high and low MET membranous expression was 18 (IQR 6 – 44) and 25 months (IQR 12 – 68) respectively (p = 0.095). The median overall survival in patients with high MET cytoplasmatic expression was 28 months (IQR 10 – 101) compared to 18 months in patients with low cytoplasmatic expression (IQR 7 – 41)(p = 0.013)). High membranous and cytoplasm expression of MET was associated with a favorable survival compared to moderate and weak expression (log rank = 0.019) (Figure 2), but cytoplasmatic expression was of greater impact in this combined analysis. When performing a subgroup analysis excluding patients undergoing neoadjuvant treatment, survival was better in patients with high membranous, or high cytoplasmatic expression, or when a combination of both was present (data not shown).

Univariable analysis of factors associated with survival showed that grade of differentiation, pT3 stage, pN stage, pM-stage, radicality of resection (R-stage), neoadjuvant chemoradiotherapy, cytoplasm MET expression and the combination of membranous and cytoplasmatic expression were significantly associated with survival. Multivariable analysis revealed that pT3, pN stage and pR-stage were independent prognostic factors for survival (Table 2).

Specificity of anti-MET antibody D1C2

In order to confirm the specificity of anti-MET antibodies, we evaluated MET gene expression in nine EAC cell lines. Gene expression of MET was detectable by qRT-PCR in all EAC cell lines with mean Ct values between 18 and 30. When normalizing to HPRT as reference gene, relative expression showed that MET was present in ESO26, P4CE, I2425, OE19, KYAE cell lines and with a high expression in ESO51 and OE33 (data not shown).
Antibody-specificity for MET was evaluated using Western blot analysis with three antibodies using a MET positive (HT29) and a MET negative cell line (LNCaP) as controls, and nine EAC cell lines (Figure 3A). Antibody D1C2, showed a specific band for MET (i.e. 145 kDa), similar to antibody 3D4 and Sp44. However, both 3D4 and Sp44 showed detection of a non-specific band around 75 kDa. Furthermore, Sp44 showed more non-specific bands in combination with more intense background expression. Also in EAC biopsy samples, MET was recognized using all three antibodies (Figure 3B). Again, in these samples Sp44 identified a clear non-specific band of around 75 kDa.

Immunohistochemistry for MET with D1C2 antibody was evaluated in a TMA of EAC and adenocarcinoma cell lines and a known positive (HT29) cell line. HT29 showed a strong membranous MET expression and a weak cytoplasmatic MET expression (Figure 3C). EAC cell lines showed differences in MET immunoreactivity with a positive membranous and cytoplasmatic expression in OE33 and ESO51, comparable to MET expression evaluated by qPCR (data not shown).

Discussion

Using D1C2 as specific antibody, MET expression was evaluated in the surgical samples of 262 EAC patients. High cytoplasm expression was associated with a better overall survival compared to weak cytoplasm MET expression. Membranous expression of MET also showed a tendency towards a better overall survival, although this correlation was not statistically significant. A combination of increased membranous and cytoplasmatic MET expression was also associated with favorable overall survival.

The present study also showed that MET expression was associated with well-known prognostic factors including TNM stage, differentiation grade and radicality of the resection [6,7,17]. Other studies in different types of cancers reported an association between MET overexpression and (favorable) clinicopathological prognostic factors such as type of tumor, T- and N-stage and histological grading [28,29].

In contrast to our findings, previous studies reported an association between MET overexpression and poor overall survival [15–17]. The present study is however in line with a more recent paper including 950 patients with gastric adenocarcinoma. MET expression was evaluated with SP44, a rabbit monoclonal antibody [18]. We evaluated MET expression using three MET antibodies by western blot, showing D1C2 to be specific for MET. This is in line with a previous study; Sp44 and D1C2 are both binding the C-terminus of the MET receptor, where D1C2 is most sensitive in the detection of membranous MET [30]. Tuynman et al. evaluated 145 patients with EAC for MET expression and found that high MET expression was associated with a worsened disease-specific survival [17]. They used a different monoclonal mouse-anti-MET antibody, named 3D4. In our study, we evaluated the specificity of D1C2 by comparing it to 3D4 and SP44 using Western blotting. All three antibodies show a specific MET band at 145 kDa in MET-negative and MET-positive cell lines and three EAC samples. However, Sp44 and 3D4 also showed a non-specific band around 75 kDa. Hence, the different results of the study by Tuynman et al. can be explained by the use of a non-specific antibody (Sp44). In addition, the smaller sample size of their study can lead to a type I error (false positive results).

Immunohistochemistry of EAC cell lines and squamous cell carcinoma cell lines with D1C2 showed abundant MET expression in EAC cells. Moreover, RT-PCR for MET expression confirmed expression in nearly all cell lines and similar results of the TMA and the RT-PCR of the OE33 and ESO51cell lines. These findings confirm that at least D1C2 is a specific antibody for MET and suggests a true positive association with a favorable survival in this large cohort of patients.

MET is a transmembrane RTK receptor and is primarily activated by its natural ligand HGF. Even though we expected that MET in its active form would primarily reside at the cell membrane, we observed in this study that MET was predominantly present in the cytoplasm and possibly masked membranous expression. An explanation for this observation could be the process of continuous endocytosis of ligand-activated MET receptors [31].

Several phase II and randomized phase III clinical trials have assessed the role of MET directed

monoclonal antibodies such as onartuzumab or rilotumumab in patients with metastatic adenocarcinoma of the gastroesophageal junction or the stomach [19]. Whereas in a randomized phase II study of rilotumumab added to standard chemotherapy increased overall survival was noted, the pivotal RILOMET-1 phase III trial, however, failed to demonstrate increased overall survival in this population [20,32]. Another phase III trial exploring the role of onartuzumab in patients with MET positive gastroesophageal cancer also showed also no superiority of adding a MET-inhibitor to standard chemotherapy [22]. These studies used different MET antibodies to investigate MET expression and to select patients for inclusion. Hence, conclusions that are drawn should be interpreted with caution.

There are limitations to this study that remain to be addressed. One of them is that only one pathologist evaluated the tumor cores for MET expression. However, this is the same pathologist who evaluated MET expression in a previous study [17]. Moreover, MET gene copy number was not assessed. However, it has been reported that only 2% of patients with gastroesophageal adenocarcinoma harbor MET amplification [13]. It is therefore questionable if gene amplification plays an important role in MET overexpression. TMA's were stained with only one antibody (D1C2).

This study was performed in a large cohort of patients with EAC, which strengthens the results. Although we stained with a relatively novel MET antibody, specificity for MET was verified by western blotting and supports the specificity of the antibody used. This study underlines that variability in sensitivity and specificity could be a hampering factor to reliably determine MET expression, thereby questioning the outcomes of clinical trials using MET directed anticancer therapies. The need and evaluation of a robust antibody is eminent given the fact that clinical trials with MET directed anticancer therapies are currently enrolling patients.

In conclusion, this study shows that in a large cohort of patients with resected EAC high cytoplasm MET expression was positively correlated with survival. Therefore, this study could serve to critically appraise current and future data of clinical studies with specific inhibitors of MET in the treatment of EAC. The search for a reliable and reproducible assessment of MET expression should continue, as until now somewhat contradictory data of MET expression in relation to clinical outcome in EAC have been presented. The assessment of the potential role of MET inhibitors in the treatment of EAC and other human tumors is currently in progress. This study is significantly adding to the discussion on the actual prognostic and/or predictive role of MET in EAC patients.

Key message

A specific MET-C terminus antibody (D1C2) was applied to a tissue micro array including esophageal adenocarcinoma biopsies. In contrast to previous observations, strong cytoplasm MET expression was associated with a favorable survival. Hence, overexpression of MET in patients with esophageal adenocarcinoma may not be a marker of poor prognosis and questions the relevance of ongoing clinical studies that select patients with MET overexpression for targeted therapy.

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Chapter three

tables and figures

Table 1

Patient and tumor characteristics according to MET expression of 262 patients with esophageal or junctional adenocarcinoma who underwent an esophagectomy.

Characteristic	All patients (n = 262)	Membrane staining		Cytoplasmic staining	
		Weak (n = 190)	**Strong (n = 72)**	**Weak (n = 93)**	**Strong (n = 169)**
Age					
Median (Range)	64 (33 – 90)				
Gender					
Male	228 (87 %)	164 (86 %)	64 (89 %)	75	153
Tumour type					
Adenocarcinoma	262 (100 %)	190 (100 %)	72 (100 %)	93 (100 %)	169 (100 %)
Tumor location					
Proximal esophagus	1 (0.4 %)	1 (0.5 %)	0 (0 %)	0 (0 %)	1 (0.6 %)
Mid esophagus	8 (3.1 %)	5 (2.6 %)	3 (4.2 %)	2 (2.2 %)	6 (3.6 %)
Distal esophagus	90 (34 %)	67 (35 %)	23 (32 %)	35 (38 %)	55 (33 %)
Gastroesophageal junction	162 (62 %)	116 (61 %)	46 (64 %)	55 (59 %)	107 (63 %)
Differentiation grade					
Good	10 (3.8 %)	5 (2.6 %)	5 (6.9 %)	6 (6.5 %)	4 (2.4 %)
Moderate	109 (42 %)	79 (42%)	30 (42 %)	36 (39 %)	73 (43 %)
Poor	138 (53 %)	102 (54 %)	36 (50 %)	51 (55 %)	87 (94 %)
Unknown	5 (1.9 %)	4 (2 %)	1 (1.4 %)	0 (0 %)	5 (5.4 %)
pT stage					
T1	27 (10 %)	13 (6.8 %)	14 (19 %)	7 (7.5 %)	20 (22 %)
T2	40 (15 %)	27 (14 %)	13 (18 %)	12 (13 %)	28 (30 %)
T3	190 (73 %)	146 (77 %)	44 (61 %)	73 (78 %)	117 (69 %)
T4	2 (0.8 %)	1 (0.5 %)	1 (1.4 %)	1 (1.1 %)	1 (0.6 %)
Tx	3 (1.1 %)				
pN-stage					
N0	72 (27.5 %)	49 (26 %)	23 (32 %)	23 (25 %)	49 (29 %)
N1	67 (25.6 %)	45 (24 %)	22 (31 %)	23 (25 %)	44 (26 %)
N2	63 (24.0 %)	47 (25 %)	16 (22 %)	27 (29 %)	36 (21 %)
N3	58 (22.1 %)	47 (25 %)	11 (15 %)	20 (22 %)	38 (22 %)
Nx	2 (0.8%)				
pM stage					
M0	251 (96 %)	180 (95 %)	71 (99 %)	91 (98 %)	160 (95 %)
M1	9 (3.4%)	8 (4.2 %)	1 (1.4 %)	2 (2.2 %)	7 (4.1 %)
Radicality					
R0	177 (68 %)	119 (63 %)	58 (81 %)	56 (60 %)	121 (72 %)
R1	85 (32 %)	71 (37 %)	14 (19 %)	37(40 %)	48 (28 %)
Neoadjuvant therapy					
Chemotherapy	29 (11 %)	26 (14 %)	3 (4.2 %)	10 (11 %)	19 (11 %)
Chemoradiotherapy	15 (5.7 %)	14 (7.4 %)	1 (1.4 %)	3 (3.2 %)	12 (7.1 %)
Radiotherapy	1 (0.4 %)	1 (0.5 %)	0	1 (1.1 %)	0 (0%)

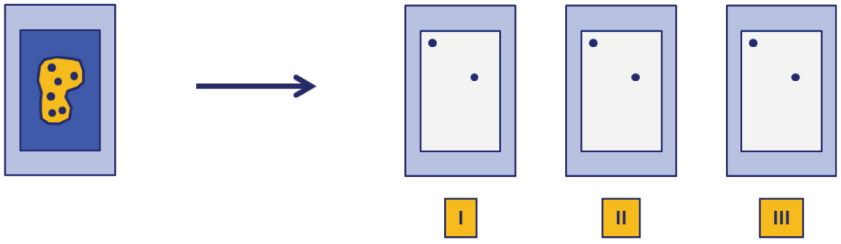
Table 2

Univariable and multivariable Cox regression analysis for five-year overall survival after esophagectomy (n=262). *Included in multivariable analyses.

	Univariable analysis Hazard Ratio (95%CI)	P-value	Multivariable analysis Hazard Ratio (95% CI)	P-value
Gender				
Man	1			
Female	0.952 (0.625 – 1.450)	0.819		
Age (yrs.)				
< 65	1			
> 65	0.808 (0.609 – 1.074)	0.142		
Differentiation grade *				
Good	1		1	
Moderate	3.602 (1.316 – 9.864)	0.013	1.702 (0.593 – 4.885)	0.323
Poor	6.052 (2.228 – 6.437)	< 0.0001	2.386 (0.827 – 6.886)	0.108
pT-stage *				
1	1		1	
2	1.781 (0.774 – 4.096)	0.175	1.230 (0.513 – 2.947)	0.643
3	5.752 (2.820 – 1.732)	< 0.0001	**2.425 (1.107 – 5.311)**	**0.027**
pN-stage *				
0	1		**1**	
1	2.422 (1.543 – 3.802)	< 0.0001	**1.730 (1.064 – 2.812)**	**0.027**
2	3.534 (2.265 – 5.514)	< 0.0001	**2.265 (1.409 – 3.641)**	**0.001**
3	6.009 (3.830 – 9.428)	< 0.0001	**3.476 (2.110 – 5.729)**	**< 0.0001**
pM-stage *				
0	1		1	
1	2.216 (1.133 – 4.337)	0.020	0.745 (0.357 – 1.556)	0.433
Radicality *				
R1	1		1	
R0	0.351 (0.261 – 0.471)	< 0.0001	**0.657 (0.467 – 0.926)**	**0.016**
Chemotherapy				
No	1			
Yes	0.730 (0.443 – 1.201)	0.215		
Chemoradiotherapy *				
No	1		1	
Yes	0.350 (0.155 – 0.790)	0.012	0.506 (0.217 – 1.179)	0.115
Radiotherapy				
No	1			
Yes	5.041 (0.695 – 6.537)	0.109		
Membranous MET *				
Low	1		1	
High	0.761 (0.573 – 1.010)	0.059	0.838 (0.569 – 1.234)	0.372
Cytoplasmic MET *				
Low	1		1	
High	0.675 (0.504 – 0.903)	0.008	0.758 (0.508 – 1.131)	0.175
Membranous and cytoplasmic MET *				
Low	1		1	
Moderate	0.754 (0.548 – 1.038)	0.083	1.006 (0.638 – 1.585)	0.980
High	0.574 (0.385 – 0.857)	0.007	1.026 (0.542 – 1.941)	0.937

Figure 1

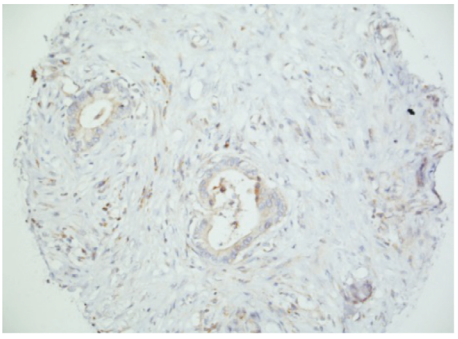
A



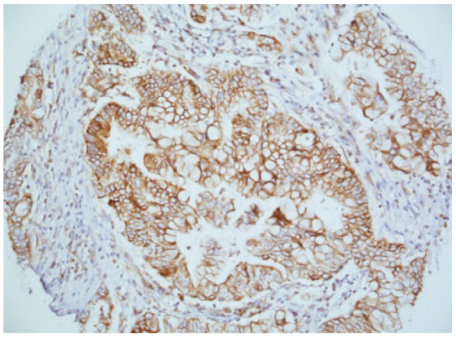
Donor block

Recipient blocks

Figure 1A:
Construction of the tissue micro array (TMA). With a 16-gauge needle four to six 0.6 mm cores from different areas of the primary tumor were taken from the FFPE blocks (donor block) and were manually bored and arrayed in a multi-tissue straw in a recognizable pattern.

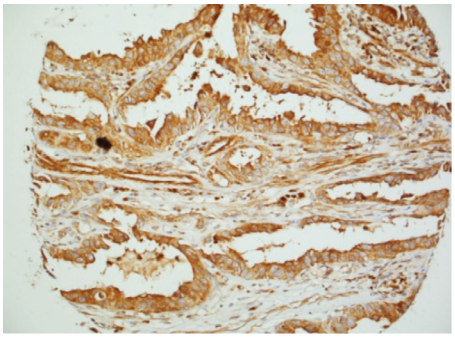


Membrane and cytoplasm negative staining

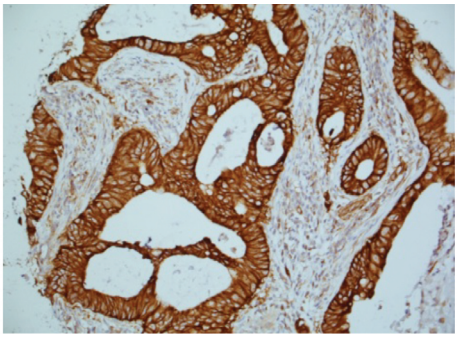


Membrane and strong cytoplasm weak staining

B



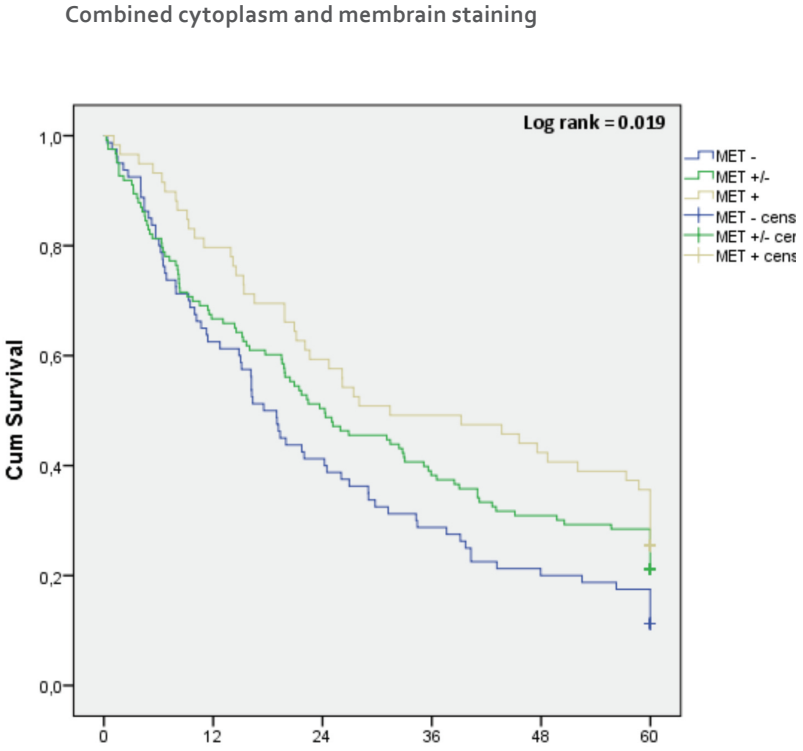
Membrane weak and cytoplasm strong staining



Membrane strong and strong cytoplasm strong staining

Figure 1B:
Staining of different tumor cores showing varied patterns of membranous and cytoplasm expression. Counterclockwise: left above membrane and cytoplasm negative staining; right above membrane strong and cytoplasm weak staining; left below weak membrane and strong cytoplasm staining; and right below strong membrane and strong cytoplasm staining.

Figure 2



Months	0	12	24	36	48	60
Low MET (N)	**79**	**49**	**32**	**22**	**15**	**13**
Moderate MET (N)	**122**	**81**	**61**	**46**	**37**	**34**
High MET (N)	**58**	**46**	**34**	**28**	**25**	**20**

Survival analysis of patients with esophageal adenocarcinoma with low (-), moderate (+/-) and high (+) combined membranous and cytoplasmatic expression (log rank = 0.019). **On the Y-axis**

Figure 3

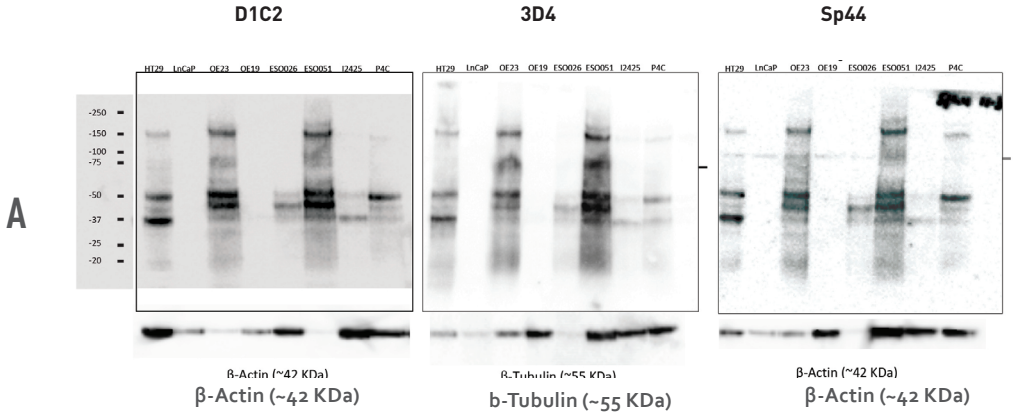


Figure 3A:
Western blot of esophageal adenocarcinoma cell lines and patient samples (B) showing a normal marker pattern with HT29 as positive control for MET showing a 145 kDa band for MET and a smaller pro-MET band just above. Three antibodies used (D1C2, 3D4 and Sp44) showing specific MET band, with in addition, 3D4 antibody showing in a panel of positive and negative MET cell lines a non-specific band of MET around 75 kDa and Sp44 showing a non-specific band around 90 kDa.

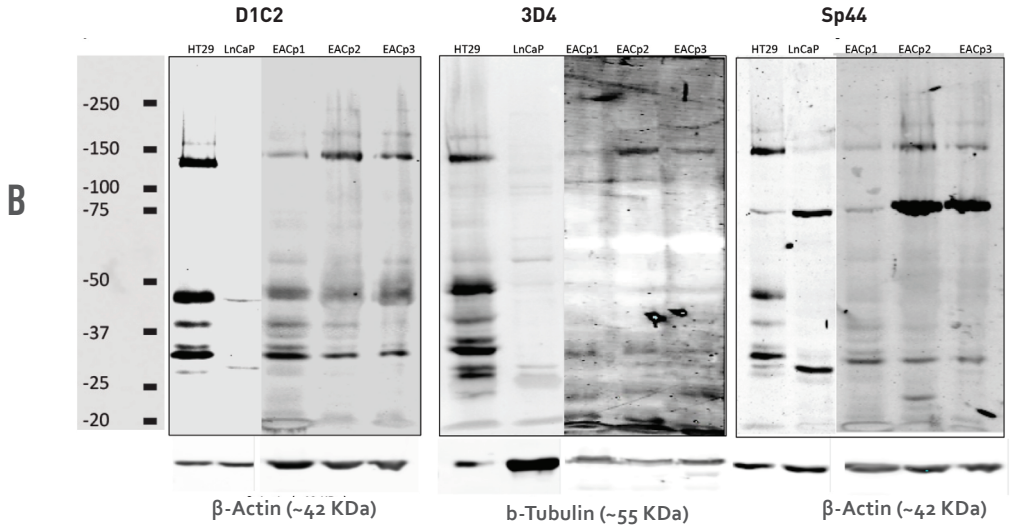
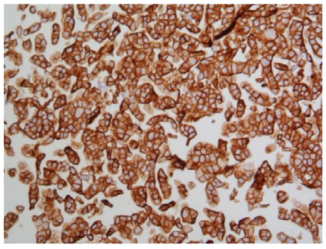
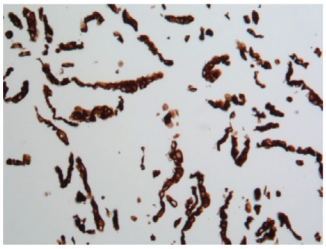


Figure 3B:
Western blot of esophageal adenocarcinoma patient biopsies showing a normal marker pattern with HT29 as positive control for MET showing a 145 kDa band for MET and a smaller pro-MET band just above. Three antibodies used (D1C2, 3D4 and Sp44) showing specific MET band, with 3D4 antibody showing in a panel of positive and negative MET cell lines a non-specific band of MET around 75 kDa and Sp44 showing a non-specific band around 90 kDa.

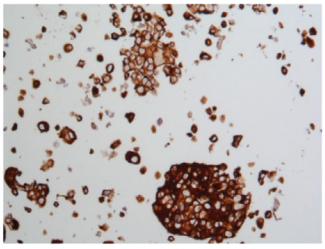
C



HT29 –strong membrane and weak cytoplasm staining



OE33 – strong membrane and strong cytoplasm staining



ESO51 – strong membrane and moderate cytoplasm staining

Figure 3C:
Immunohistochemistry on TMA of EAC cell lines with D1C2: HT29 as positive control for MET, OE33 and ESO51 stained positive with D1C2.





Chapter four

MicroRNA-126 controls tumour cell viability and is associated with poor survival in patients with oesophageal adenocarcinoma

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Exp. Biology and Medicine, 2019, in press

Abstract

BACKGROUND

Oesophageal adenocarcinoma (OAC) displays a poor prognosis and current treatments are often not curative. Pathological TNM-stage is a prognostic parameter, but a better understanding of the pathophysiology of OAC is needed to better predict survival. Recent work in other malignancies indicated an important role for the regulator microRNA- 126 (miR-126) in tumours. The aim of this study was to investigate the function of miR-126 in OAC and to correlate expression of miR-126 with tumour cell behaviour and patient survival.

MATERIALS AND METHODS

Functional assays were performed in OAC cell lines (OE33) in vitro by overexpressing or antagonising miR-126 and assessing cellular processes linked to the hallmarks of cancer. In vivo pre-treatment biopsies of 58 patients with OAC who underwent neoadjuvant chemoradiotherapy and surgery were analysed for miR-126 expression in tumour cells by qRT-PCR and patient survival was analysed by Kaplan Meier and Cox regression.

RESULTS

In OE33 cancer cells stable overexpression of miR-126 modest though significantly altered expression of genes related to cell death (MEK1) and DNA repair (POLB and TERF1) was observed. Also the secretion of the angiogenic and pro-inflammatory factors, VEGF, IL-1β and IL-6 were regulated by miR-126 ($p<0.029$). Importantly, miR-126 was found to be a regulator of cell viability in OE33 cells. Overexpressing ($p=0.043$) and antagonising ($p=0.035$) miR-126 showed reciprocal effects on tumour cell viability and significantly regulated expression of pro- and anti-apoptotic genes, TP53 and GATA6 ($p<0.031$). In patients, high levels of miR-126 expression in pre-treatment tumours was significantly associated with poor survival ($p=0.031$). In multivariable analysis, high miR-126 ($p=0.038$) together with ypN-stage ($p=0.048$) were shown to be independent risk factors for poor survival.

CONCLUSION

In conclusion, high expression of miR-126 in OAC prevents tumour-cell death and is associated with poor patient survival. This study warrants further analysis of miR-126 as biomarker or potential therapeutic target for OAC.

Impact statement

Oesophageal adenocarcinoma is a common form of cancer of the oesophagus. It has an increasing health impact as it is associated with very poor patient survival. A better understanding of the pathophysiology of this cancer is needed to identify better treatment strategies and to provide a better prognosis for these patients. MicroRNAs have emerged as important molecular regulators of cancer cell viability and proliferation. The aim of our study was to investigate the role of one very well established microRNA, miR-126, in oesophageal adenocarcinoma. Our research shows clear experimental evidence that miR-126 controls cell viability of oesophageal adenocarcinoma cells. High (over)expression of miR-126 increased the viability of these cells. Our preclinical data were shown to be clinically relevant for this field of oncology. In an independent validation study of oesophageal adenocarcinoma biopsies, we confirmed that high miR-126 expression in tumour cells was an independent risk factor for poor patient survival.

Introduction

Cancers of the oesophagus are often diagnosed at an advanced stage, which results in less than half of the patients being eligible for potentially curative treatment at diagnosis [1]. Incidences of oesophageal cancer, and especially of oesophageal adenocarcinoma (OAC) are rising in Western Europe [2]. Neoadjuvant chemoradiotherapy (nCRT) followed by surgery is considered standard treatment for locoregional disease (cT1N1 and cT2-4a, cN0-N3, M0). Multimodality treatment is able to downstage the tumour, it facilitates a resection with tumour negative margins and may cure (locoregional) micrometastases at an early stage. Recently, the CROSS trial showed that these expectations are achieved and that patients who underwent nCRT followed by surgery had a better survival and had reduced locoregional recurrences as compared to surgery alone [3–6]. There is an ongoing need to identify objective biomarkers to predict response to neoadjuvant chemoradiotherapy or to predict survival. Currently, a promising approach for molecular characterisation of tumours is the understanding of the role that microRNAs (miRNAs) play in cancer.

MiRNAs are small non-coding RNAs who are able to regulate gene expression. Approximately one-third of all human genes are directly regulated by miRNA [7,8]. The miRNA, by interaction with a complementary sequence in mRNA, causes inhibition of post-transcriptional translation or induces targeted mRNA degradation [9,10]. MiRNAs regulate many important cellular processes such as cell proliferation, migration and apoptosis. Dysregulation of miRNAs occurs in a number of pathological conditions most prominent in cancer [7,8,11].

Several cancer associated miRNAs have been now identified, of which miRNA-126 (miR-126) is one of the most established and broad acting ones. MiR-126 is implicated to play an important role in cancer biology of different cancer types including breast-, gastric- and pancreatic neoplasms [12]. Two pathways that have been linked with miR-126 are angiogenesis and cell death [13,14]. More recently a role in mitochondrial function and metabolism has been demonstrated [15,16]. In non-small cell lung cancer patients, low miR-126 expression in either biopsies or resection specimens was correlated with a poorer survival compared to tumours with high expression [17]. In these tumours, high miR-126 expression, combined with VEGF co-expression, is prognostically unfavourable for patients and points more towards an oncogenic role [18]. Furthermore, in patients with colon cancer high expression of miR-126 in the resection specimen is related to a better overall survival [19].

The role of miR-126 in oesophageal cancer is less well established. Three known target genes of miR-126, namely VEGFA, TP53 and GATA6, are often amplified in the genome of OAC tumour cells [20,21]. In squamous cell carcinoma, miR-126 expression was decreased when compared to normal squamous epithelium in the oesophagus [22]. In OAC resection specimens, miR-126 expression is associated with poor prognostics factors including tumour cell dedifferentiation and lymphatic dissemination [23]. The exact impact of miR-126 expression on the prognosis of survival in patients with OAC remains yet to be established. Moreover, it is still unknown which cancer signalling pathways are involved. The aim of this study is to investigate the functional role of miR-126 in OAC cells and profile if the level of miR-126 has associations with patient survival.

Materials and methods

PATIENTS, DISEASE STAGING AND TREATMENT

Some 58 patients with histologically proven adenocarcinoma of the intrathoracic oesophagus or gastro-oesophageal junction who underwent neoadjuvant chemoradiotherapy followed by surgery were identified from a prospectively collected institutional database. All patients were treated at the Erasmus MC University Medical Centre Rotterdam, which is a tertiary referral centre for patients with oesophageal carcinoma in the Netherlands. The use of tissue biopsies for this research has been approved by the local medical ethical committee at the Erasmus MC Rotterdam and was judged not WMO obligatory. Tumours were staged according to the 7th UICC-AJCC TNM staging manual [24]. An upper gastro-intestinal endoscopy with biopsies, endoscopic ultrasonography (EUS) with fine needle aspiration (FNA) when indicated, external ultrasonography of the neck with FNA and computed tomography (CT) of the neck, chest and abdomen was performed in every patient. Bronchoscopy was only indicated for tumours of the intrathoracic oesophagus when there was suspicion of infiltration of the tracheobronchial tree. Patients received chemoradiotherapy prior to surgery according to CROSS [5]. In short, carboplatin and paclitaxel were administered intravenously on a weekly basis consisting of five courses with concurrent irradiation with 23 fractions of 1.8 Gy each. Surgical resection of the oesophagus was performed within 4 to 6 weeks of completing the nCRT regimen. Patient's survival was calculated from date of surgery till date of death or date of follow up, updated in March 2015, irrespective of cause of death.

TISSUE HANDLING AND LASER CAPTURE MICRODISSECTION

Pre-treatment formalin fixed paraffin embedded (FFPE) biopsies were requested from the tissue-bank in the Erasmus Medical Centre Rotterdam. A rotation microtome was used to cut the biopsies to 4 µm thickness. The first two slides and the last one were collected on normal Star-Frost glass-slides to identify the tumour locations before laser capture micro dissection. The in between slides were collected and placed on to special membrane slides (Zeiss Membraneslide 1.0 PEN, Zeiss, Breda, the Netherlands). The next day these slides were deparaffinised and shortly (1-2 seconds) stained with haematoxylin (Mayer's hemalum solution for microscopy), followed by a short rehydration through graded concentrations of ethanol in water. After drying of the slides, the tumour areas in the biopsies were selected by a pathologist before the laser capture micro dissection.

Laser capture microdissection was performed with the PALM laser micro dissector (Carl Zeiss Micro Imaging, Breda, The Netherlands). The micro dissected tumour pieces were collected in the cap of a 500 µL autoclaved tube within a drop (40 µl) of digestion buffer (RecoverAll Total Nucleic Acid Isolation Kit for FFPE, Applied Biosystems, Fisher Scientific, Landsmeer, the Netherlands). More digestion buffer (160 µL) was added, together with 4 µL protease. The tubes were placed in a micro centrifuge and spinned shortly to collect all the liquid with tissue to the bottom of the tube. The samples were then incubated in a heat block for 15 minutes at 50°C, followed by 15 minutes at 80°C and stored at -20°C.

RNA-ISOLATION AND QUANTITATIVE REVERSE TRANSCRIPT PCR (QRT-PCR)

RNA isolation was performed according to the manufacturer's instructions (RecoverAll Total Nucleic Acid Isolation Kit for FFPE from Applied Biosystems, Fisher Scientific, Landsmeer, the Netherlands). MicroRNA-specific cDNA was synthesised using a Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) containing for every reaction 0.4 µl 100 mM dNTP's, 1.35 µl multiscribe RT, 2.0 µl 10x RT Buffer, 0.25 µl RNase inhibitor and 1.0 µl of the primer for miR-126 (UCGUACCGUGAGUAAUAAUGCG) and RNU43 as endogenous control for data normalisation. Real time qRT-PCR was carried out in duplicate and each reaction consisted of 10 µl TaqMan Universal PCR Master Mix (TaqMan® Universal PCR Master Mix No AmpErase® UNG), 0.5 µl miR specific PCR primer (Applied Biosystems) and 5.0 µl of diluted cDNA. The reaction ran in a 40 cycle-schedule: 10 min at 95°C, followed by 40 times 15 seconds 95 C for 1 min 60 °C (iQ5 multicolour real-time PCR detection system Bio-Rad Laboratories, Veenendaal, the Netherlands).
MiR-126 expression was quantified based on the 2-ΔCt method where relative levels were normalised to levels of reference non-coding RNA, RNU43. Groups were created based on expression of miR-126, where a cut-off was made at the 75th percentile to discriminate high (top25%) versus low expression.

TRANSFECTION OF CELL LINES

To establish a stable cell line overexpressing miR-126, OE33 cells were transfected via Lipofectamine (Applied Biosystems) with 10 ng of the plasmid carrying the miR-126 sequence 5'-CGUACCGUGAGUAAUAAUGCG-or an empty plasmid as control (vector control) (OriGene, Herford, Germany). Continuous selection was carried out with 0.5 mg/ml G418 (Sigma-Aldrich, Zwijndrecht, the Netherlands) added to the cell culture media after 24 hours after transfection. G418 resistant clones were maintained in RPMI (+ 10% FCS) with 0.5 mg/ml G418. MiR-126 expression levels were measured via qRT-PCR analysis normalised to RNU43 as reference gene, to evaluate the degree of overexpression per experiment. A transient overexpression of miR-126 in OE33 cells was established within 48 hours with similar transduction except without continuous selection. To antagonise miR-126, OE33 cells were transiently transfected after overnight adherence with 5 µM of antimiR-126 or a scrambled control (both fluorescein-labeled, Exiqon, QIAGEN Benelux B.V. Venlo The Netherlands) using Dharmafect I (Dharmacon, Cambrigde, United Kingdom). Transfection efficiency was measured after 24 hours with flow cytometry.

GENE EXPRESSION ANALYSIS IN OE33 (CANCER PATHWAYS)

To screen which cancer pathways miR-126 was altering, a cancer pathway finder pathway gene array analysis was performed which included 84 genes, representing nine cancer pathways (angiogenesis, apoptosis, cell cycle, cellular senescence, DNA damage and repair, epithelial to mesenchymal transition, hypoxia signalling, metabolism, and telomeres and telomerase) (RT2 Profiler PCR Array, Qiagen, Manchester, UK). Three independent batches of paired stably overexpressing miR-126 and accompanying empty vector controls were cultured and RNA was isolated from the cells. cDNA was made according to the manufacturers protocol (Bioscript, Berlin, Germany) from 1 µg input RNA. The qRT-PCR analysis was performed with a customised SYBR green master mix according to the manufacturer's protocol and ran for 40 cycles (Applied Biosystems 7900 HT Fast Real-Time PCR system) to obtain relative expression levels.
Based on the results of the cancer pathway analysis, validation of the expression levels of the fol-

lowing genes was conducted by q-RT-PCR: CDH1, CTNNBL1, SNAI1, SNAI2, TWIST1 and ZEB2 for epithelial to mesenchymal transition (EMT), SMUG1, PARP1, MLH1 and MMS19 for DNA damage and repair. 18S was used as a reference gene. Furthermore pro-apoptotic gene TP53 and anti-apoptotic gene GATA6 were measured in each of the samples by qRT-PCR [21,25,26].

ELISA DETECTION OF INFLAMMATION AND ANGIOGENESIS FACTORS

In order to assess if miR-126 affected the secretion of inflammatory and angiogenic proteins, MSD Multispot ELISA were performed (Meso Scale Diagnostics, Rockville, Maryland, USA). The inflammatory panel included IL-1β, IL-2, IL-10, IL-6, MCP-1, MIP-3a, Gro-a, MMP2, MMP9 and TNF-α. The angiogenic panel included VEGF, Ang-1, Ang-2, bFGF, PAI-1, sVCAM and sICAM. Conditioned culture medium of untransfected and transfected OE33 cells was analyzed using this multiplex panel according to the manufacturer's instructions.

FLOWCYTOMETRY

After 24 hours of transient antagonising transfection cells were harvested and 7AAD (BD Biosciences, Vianen, the Netherlands) was added prior to flowcytometry. Controls were taken in to account for compensations (OE33 cells unstained, OE33 cells + 10% DMSO + 7 AAD for single staining in Per-Cyp-5 channel and OE33 cells + antimiR-126 for single staining in FITC channel). By adding 7 AAD apoptosis was measured for transfected cells in the FITC channel. Similar to this method transient overexpressing transfected cells were analysed for cell death.

STATISTICAL ANALYSIS

The probability of survival over time was estimated with the Kaplan–Meier method and the log-rank test was used to determine statistical differences between groups. To determine which variables affect survival, all variables with a significance $P < 0.100$ together with clinically relevant variables were included in a multivariable logistic regression model. Statistical significance was set at the 5% level. Statistical analysis was performed with the use of SPSS software, version 22.0 (SPSS, IBM, New York, USA).

Results

MIR-126 OVEREXPRESSION PROVIDE SUBTLE CHANGES IN MAP KINASE GENE EXPRESSION AND CYTOKINES & VASCULAR GROWTH FACTOR PRODUCTION IN OAC CELLS.

Using OE33 oesophageal cancer cells stably overexpressing miR-126, a cancer pathway analysis was performed which included 84 specific genes representing nine different cancer pathways including programmed cell death, cell cycle, DNA damage and repair, angiogenesis and epithelial to mesenchymal transition (EMT). As shown in Figure 1A, overall effects of miR-126 overexpression were relatively modest and significance in gene expression was detected for three genes. As shown in Figure 1B, MAP kinase MEK1 was significantly downregulated in samples overexpressing miR-126 compared to its empty vector control ($p=0.011$). The MEK1 MAP kinase pathway is a known regulator of cell death in various tumour cell types. This effect was not validated by conventional RT-PCR as the exact sequence of these PCR primers is not disclosed. Additional findings from cancer pathway analysis were two genes related to DNA damage responses, POLB (DNA polymerase beta) and TERF1 (telomeric repeat binding factor 1) (Fig. 1B). Based on these results, further validation of selected genes related to epithelial to mesenchymal transition (EMT) and DNA damage responses was performed by targeted qRT-PCR. In this validation none of the EMT or DNA damage response genes were significantly regulated by miR-126 (data not shown). Further, the effect of miR-126 on the production and secretion of pro-inflammatory and angiogenic factors by EO33 cells was evaluated using a multispot ELISA essay. Medium of control OE33 cells and miR-126 overexpressing cells were tested for a panel of ten pro-inflammatory factors and seven angiogenic factors. Of all angiogenic factors, only proangiogenic factor VEGF was significantly lower in OE33 cells stably transfected with miR-126 (25% reduction, $p=0.009$ paired t test, data not shown). Furthermore, levels of the two pro-inflammatory cytokines IL-1β and IL-6 were significantly higher in miR-126 overexpressing OE33 cells (97% and 112% increase respectively, $p<0.029$ paired t test, data not shown).

MIR-126 AFFECTS CELL DEATH AND REGULATES CELL-DEATH RELATED GENE EXPRESSION IN OAC CELLS.

To further investigate the role of miR-126 in regulating programmed cell death, quantification of cell vitality and cell death was performed using flow cytometry. To this end, OE33 cells were transiently transfected with a plasmid containing miR-126 or an empty vector control. As shown in Figure 2A, there was less death of OE33 cells overexpressing miR-126. The reverse effects were seen when OE33 cells were transfected with an antimiR-126 resulting in a significantly increased death of OE33 cells as compared to cells transfected with a non-specific antimiR control (Figure 2B). This effect was only observed in cells with detectable antimiR levels as shown by fluorescein labelling.
Subsequently, gene expression analysis was performed for the pro- and anti-apoptotic genes TP53 and GATA6. As shown in Figure 3, OE33 cells transfected with an antimiR-126 showed increased mRNA levels of the pro-apoptotic tumour suppressor gene TP53 ($p=0.015$). This indicates that miR-126 downregulates expression of TP53 in OAC. OE33 cells transfected with an antimiR-126 showed a significant decreased mRNA level of the anti-apoptotic gene GATA6, as compared to control treated cells ($p=0.031$). This indicates that miR-126 indirectly upregulates expression of GATA6 in this OAC cell line. Together, these data indicate that miR-126 may be an important regulator of cell death in this OAC cell line model.

HIGH MIR-126 EXPRESSION IN TUMOUR IS ASSOCIATED WITH POOR PATIENT SURVIVAL.

Next we evaluated the role of miR-126 in OAC patient samples, all treated with neoadjuvant chemoradiotherapy followed by surgery (Figure 4A). These samples were analysed for miR-126 expression levels in their baseline pre-treatment biopsies. Laser capture microdissection was performed in order to obtain tumour specific RNA (Figure 4B). Pre- and post-treatment patient and tumour characteristics are shown in Table 1. The majority of the patients were down staged by the neoadjuvant chemoradiotherapy, where 34% had a pathologically complete response. As shown in Figure 4C, the relative expression of miR-126 in the laser capture tumour tissue greatly varied per patient. In tumour tissue with the lowest miR-126 level (lowest 25%), the relative expression in the complete biopsy section, which included RNA from both tumour and non-tumorous squamous epithelial cells, was a mean 16.3-fold higher than tumour alone ($p=0.002$). In contrast, in tumours with the highest relative miR-126 levels (top 25%) there was no significant difference compared to the complete samples containing both tumour and squamous epithelial cells ($p=0.08$). This suggests that the average level of miR-126 in the majority of OAC tumour cells is reduced compared to non-tumorous oesophagus squamous epithelial cells.

In fourteen patients with high relative expression of miR-126 (top 25%) the average expression level was 2.3-fold higher compared to 44 patients with a low relative expression (Figure 5A). Tumour or whole biopsy miR-126 expression was not predictive of response to neoadjuvant therapy with 95% CI including one for all four tumour regression grades (data not shown). The median five year survival after surgery of all 58 patients was 36 months (IQR). No statistically significant association was observed between miR-126 levels in total biopsy sections and patient survival (data not shown). However, for tumour-specific miR-126 levels, patients with high miR-126 expression had a median survival of 14 months (IQR 37 months) compared to 41 months (IQR 39 months) for patients with low relative expression of miR-126 in tumours (Log rank $p=0.031$, Figure 5B). Univariable and multivariable analysis of pre- and on-treatment characteristics which affect survival are shown in Table 2. The pre-treatment tumour miR-126 levels and the T stage (ypT) and N stage (ypN) (both derivated from the resection specimen) were all associated with five year survival. Also post-surgery recurrence of disease significantly affected survival (HR 12.9, $p < 0.0001$, not shown). The relatively low number of patients limited the number of variables to be included in multivariable analysis. Taken the three most significant univariables, only pre-treatment miR-126 (HR 2.5, $p=0.038$) and N-stage (ypN2, HR 4.6, $p=0.048$) were independent risk factors for poor survival in multivariable analysis (Table 2). Taken together these data indicate that high miR-126 levels in tumour cells is associated with a more progressive cancer resulting in reduced patient survival despite neoadjuvant chemoradiotherapy and oesophagectomy treatments.

Discussion

In this study the function and expression of miR-126 in OAC was assessed in vitro and in vivo. The results show a role for miR-126 in regulating tumour cell death and that high miR-126 expression is associated with poor survival. The first step in this process was to create an OE33 cell line overexpressing miR-126 by stable transfection. VEGF production and secretion was significantly downregulated by miR-126. This is in line with previous studies, where overexpression of miR-126 mimics causes a downregulation of VEGF in ovarian, hepatocellular and lung cancer [27–30]. With the cancer pathway finder, testing 9 different cancer pathways included total 84 specific genes, we found that only MAP kinase (MEK1) and DNA damage (POLB and TERF1) genes were slightly though significantly affected by miR-126 overexpression in univariable analysis. Interestingly, a recent study showed that miR-126 (mimic) increase telomere length, consistent with the observed increase of TERF1 gene expression in EO33 cells (Fig. 1) [31]. In statistics, the problem of multiple comparisons, like in the case for the cancer pathway assay, the Bonferroni correction or related methods have been proposed. However, tests like the Bonferroni correction reduce the statistical power and come at the cost of increasing the probability of producing false negatives. There is not a definitive consensus on how to correct for multiplicity and is therefore not used in our analyses.

The exact mechanism by which miR-126 regulated expression of these genes is not clear and may involve either direct or indirect effects. Direct regulation involves the miRNA binding to the 3'-UTR of the mRNA molecule suppressing the translation or promoting degradation. Indirectly regulation would involve interaction of miR-126 with a mRNA of regulatory proteins, like transcription factors or signalling molecules, indirectly suppressing down-stream genes. Indeed, some of the gene regulation is likely to be indirectly, as the mRNA levels of TERF1 (Fig. 1) and GATA6 (Fig. 3) are increased, rather than suppressed, this would suggest indirect regulation by miR-126. This can be explained by miR-126 suppressing the translation of regulatory proteins that normally suppress the expression of TERF1 or GATA6. Regarding the targeting of the 3'-UTR of genes by miRNAs, bioinformatics databases are available. According to the Targetscan database (http://www.targetscan.org/) [32], TERF1, VEGFA and IL-6 are confirmed targets of miR-126. Related to the increased production of IL-1β by miR-126 overexpression in EO33 cells, the type I interleukin 1 receptor (IL1R1) and the interleukin 1α (IL1A) genes are predicted targets of miR-126 and maybe involved in indirect regulation of IL-1β.

In the cancer pathway analysis, particular MEK1 showed a statistically significant lower expression in cells where miR-126 was overexpressed compared to the vector control. According to bioinformatics data, MEK1 is not a direct target of regulation by miR-126. However, miR-126 is a predicted target of the SMEK1 and SMEK2 genes which may indirectly effect MEK1 [32]. MEK1, also known as MAP2K1, was earlier identified as a factor in the development of cancer in general, but also in OAC [33,34]. MEK1 is an essential component of the MAP kinase signal transduction pathway, regulating among others cellular homeostasis and apoptosis [35]. When miR-126 was overexpressed in hepatocellular carcinoma cell lines expression of EGFL7, ERK, Bcl-2, and P-ERK was suppressed, and expression levels of apoptotic-associated proteins Fas/FasL and Caspase-3 were increased. Furthermore it induced apoptosis and inhibited cell proliferation [36]. It is known that cancer cells are resistant to programmed cell death and can exhibit cellular senescence [37].

Regarding the effects of miR-126 on OAC cell death, in the experiments where miR-126 was transiently overexpressed or antagonised, miR-126 showed reciprocal effects on tumour cell death. Indeed, in earlier studies miR-126 is linked to apoptosis in the setting of rheumatoid ar-

thritis by inhibiting the PI3K/AKT signal pathway and thereby inhibiting apoptosis [30,31,38,39]. Furthermore miR-126 significantly affected cell death regulating genes, TP53 and GATA6 [21,25,31]. Regarding the role of TP53, a recent study showed in kidney mesangial cells showed that miR-126 overexpression reduced TP53 protein expression [31]. In accordance with this observation, in OE33 cells TP53 gene expression was increased by antagonizing miR-126 by antimiR-126 (Fig 3). Though there is no evidence that miR-126 directly targets the 3'-UTR of TP53 mRNA, several TP53-related genes are predicted targets. These include TP53 apoptosis effector (PERP), TP53 inducible ribonucleotide reductase (RRM2B), TP53 inducible nuclear protein (TP53INP1), and TP53 regulating kinase (P53RK) [32]. Regarding the role of GATA6, an earlier study showed that silencing of GATA6 induces apoptosis in OAC cells but not in oesophageal squamous cells. Moreover, OAC patients whose tumours carry a GATA6 gene amplification showed poorer survival [21]. Finally, there is new compelling evidence for a role of IL-6 signaling in OAC cell death [40]. Using a xenografting model of OE33 cells in mice, it was showed that anti-IL-6Rα antibody, Tocilizumab, suppressed tumour growth in vivo. This tumour suppression was associated with increased cell death of OE33 cells in vivo as shown by increased cleaved-caspase 3 staining. These findings are consistent with our in vitro results, showing that overexpression of miR-126 in OE33 cells increased IL-6 production and reduced cell death. Further experiments would need to show the role of IL-6 signalling in relation to cell death in miR-126 overexpressing OE33 cells.

To identify the clinical relevance of the possible role of miR-126 in tumour cell viability, patients diagnosed with OAC, treated with curative intent with neoadjuvant chemoradiotherapy followed by surgery were selected. In their pre-treatment biopsies tumour-RNA was analysed for miR-126 expression. Patients with a relatively higher expression of miR-126 had significantly worse prognosis than patients with lower expression of miR-126. These results were in line with the in vitro experiments with OE33 cells, showing that high (over)expression of miR-126 was associated with less cancer cell death in a cell viability assay (Fig. 2). Both TNM-stage and miR-126 expression were significantly associated with patient survival (Table 2).

When comparing our findings to studies performed in other cancer types, somewhat contradictory results are observed. For instance in non-small cell lung cancer patients low miR-126 expression, in either biopsies or resection specimens, was correlated with a poorer survival compared to tumours with high expression [17]. Similarly, in patients with colon cancer, high expression of miR-126 in the resection specimen was correlated to a better overall survival [19]. These differences may be explained by a different biological role of miR-126 in these cancers and a difference in methodology used for miRNA quantification. On the other hand, our results are supported by an earlier study looking at the relationship between miRNA expression and survival in the patients with OAC. Using in situ hybridization with digoxigenin-labeled miRNA probes on tumour microarrays from nearly 100 patients, high expression of miR-126 was significantly associated with tumour cell dedifferentiation and lymph node metastasis, and with a non-significant trend towards poorer overall patient survival [23]. Further prospective studies are required to validate these observations and show the exact role miR-126 on disease progression in patients with OAC.

Although this study supports a role of miR-126 in regulating cell viability in oesophageal adenocarcinoma, there are several limitations to address. An adequate multivariable analysis was limited due to the sample size, however it revealed miR-126 as an independent factor influencing survival. It was unfortunately not feasible to enlarge the group or to validate the results in an independent cohort, due to the limited availability of tissue for tumour-RNA isolation (laser capture microdissection). In vitro data could not be easily compared to other studies where miR-126 was overexpressed or silenced in different cancer types. This study focussed on different

pathways to elucidate the in vivo findings and makes it therefore a valuable result to share. In conclusion, this is the first study demonstrating a link between miR-126 and cell viability in oesophageal adenocarcinoma and this warrants further study of miR-126 as biomarker or potential therapeutic target for OAC.

Author Contributions:
All authors participated in the design of the study and editing of the manuscript; ELAT, NLL, KB, GD and PER conducted the experiments, analyzed and interpreted data, KB, JJBL, JVR and BPLW provided clinical input, ELAT JOS and LJWL wrote the manuscript, JOS and LJWL supervised the project.

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Declaration of Conflicting Interests:
There a no conflicts of interest.

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Chapter four

tables and figures

Table 1

Patient and tumour characteristics at baseline and post-surgery of 58 patients receiving neoadjuvant chemoradiotherapy followed by surgery.

Characteristic	Pre-treatment	Post-surgery
Gender (male)	50 (86.2 %)	50 (86.2 %)
Age (mean (range))	60 (36 – 78)	60 (36 – 78)
Histology (AC)	58 (100%)	38 (65.5 %)
Location		
Mid	5 (8.6 %)	-
Distal	34 (58.6 %)	-
Gastro-oesophageal junction	19 (32.8 %)	-
(yp)T-stage		
0	0	20 (34.5 %)
1	0	9 (15.5 %)
2	20 (34.5 %)	6 (10.3 %)
3	37 (63.8 %)	20 (34.5 %)
4	1 (1.7 %)	0
Missing	0	3 (5.2%)
(yp)N-stage		
0	17 (29.3 %)	40 (69 %)
1	25 (43.1 %)	12 (21 %)
2	15 (25.9 %)	6 (10 %)
3	1 (1.7 %)	
(yp)M-stage		
0	58 (100 %)	57 (98.3%)
1	0	1 (1.7%)
Grading		
Good	2 (3.4 %)	1 (1.7 %)
Moderate	33 (56.9 %)	22 (37.9 %)
Poor	21 (36.2 %)	10 (17.2 %)
Missing	2 (3.4 %)	25 (43.1 %)
Radicality		
R0	-	53 (91 %)
R1	-	5 (9 %)
Tumour regression grade		
TRG 1	-	20 (34.5 %)
TRG 2	-	12 (20.7 %)
TRG 3	-	15 (25.9 %)
TRG 4	-	11 (19.0 %)
Recurrence		
No	-	34 (58.6 %)
Yes	-	24 (41.4 %)

Abbreviations:
AC = adenocarcinoma;
yp = after neoadjuvant treatment, after resection,
p = pathology;
R0, R1 = radicality of the resection;
TRG = tumour regression grade.

Table 2

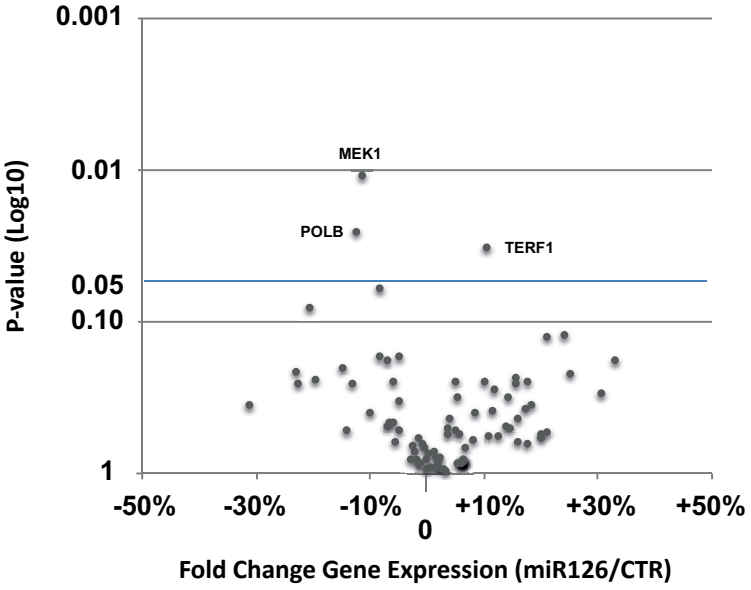
Univariable (UV) and multivariable (MV) analysis pre- and post-treatment characteristics affecting five year survival of 58 patients receiving neoadjuvant chemoradiotherapy followed by surgery.

Parameters	HR (95% CI), p value, UV	HR (95% CI), p value, MV
Gender		
Male	1 (Ref)	x
Female	0.664 (0.201 – 2.191), p = 0.502	x
Age		
< 65 year	1 (Ref)	x
≥ 65 year	1.698 (0.817 – 3.531), p = 0.156	x
Location		
Mid	1 (Ref)	x
Distal	0.406 (0.116 – 1.471), p = 0.157	x
Junction	0.809 (0.230 – 2.845), p = 0.741	x
ypT-stage *		
ypT0	1 (Ref)	1 (Ref)
ypT1	1.607 (0.470 – 5.495), p = 0.449	0.880 (0.950 – 8.154), p = 0.910
ypT2	1.010 (0.210 – 4.871), p = 0.990	0.617 (0.056 – 6.808), p = 0.693
ypT3	**3.965 (1.611 – 9.755), p = 0.003**	2.019 (0.435 – 9.365), p = 0.369
ypN-stage *		
ypN0	1 (Ref)	1 (Ref)
ypN1	1.245 (0.494 – 3.139), p = 0.642	1.299 (0.385 – 4.389), p = 0.673
ypN2	**6.336 (2.015 – 19.923), p = 0.002**	**4.606 (1.013 – 20.936), p = 0.048)**
ypN3	**4.917 (1.085 – 22.273), p = 0.039**	1.995 (0.334 – 11.898), p = 0.449)
Radicality		
R0	1 (Ref)	x
R1	2.506 (0.870 – 7.218), p = 0.089	x
Tumour regression grade		
TRG 1	1 (Ref)	x
TRG 2	2.453 (0.859 – 7.008), p = 0.094	x
TRG 3	2.234 (0.829 – 6.020), p = 0.112	x
TRG 4	2.637 (0.921 – 7.551), p = 0.071	x
MiR-126 expression *		
Low	1 (Ref)	1 (ref)
High	**2.259 (1.053 – 4.846), p = 0.036**	**2.558 (1.052 – 6.220), p = 0.038**

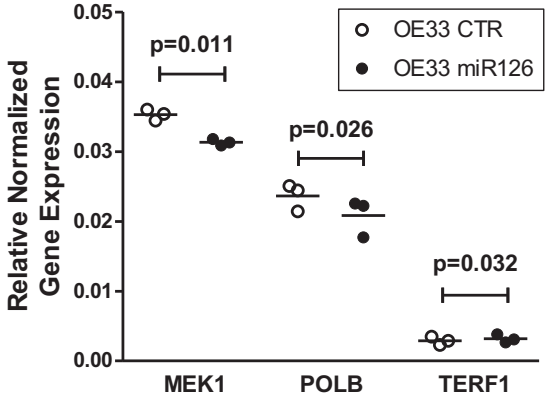
Abbreviations:
yp = after neoadjuvant treatment, after resection,
p = pathology;
R0, R1 = radicality of the resection;
TRG = tumour regression grade; * = taken into account for multivariable analysis.

Figure 1

A

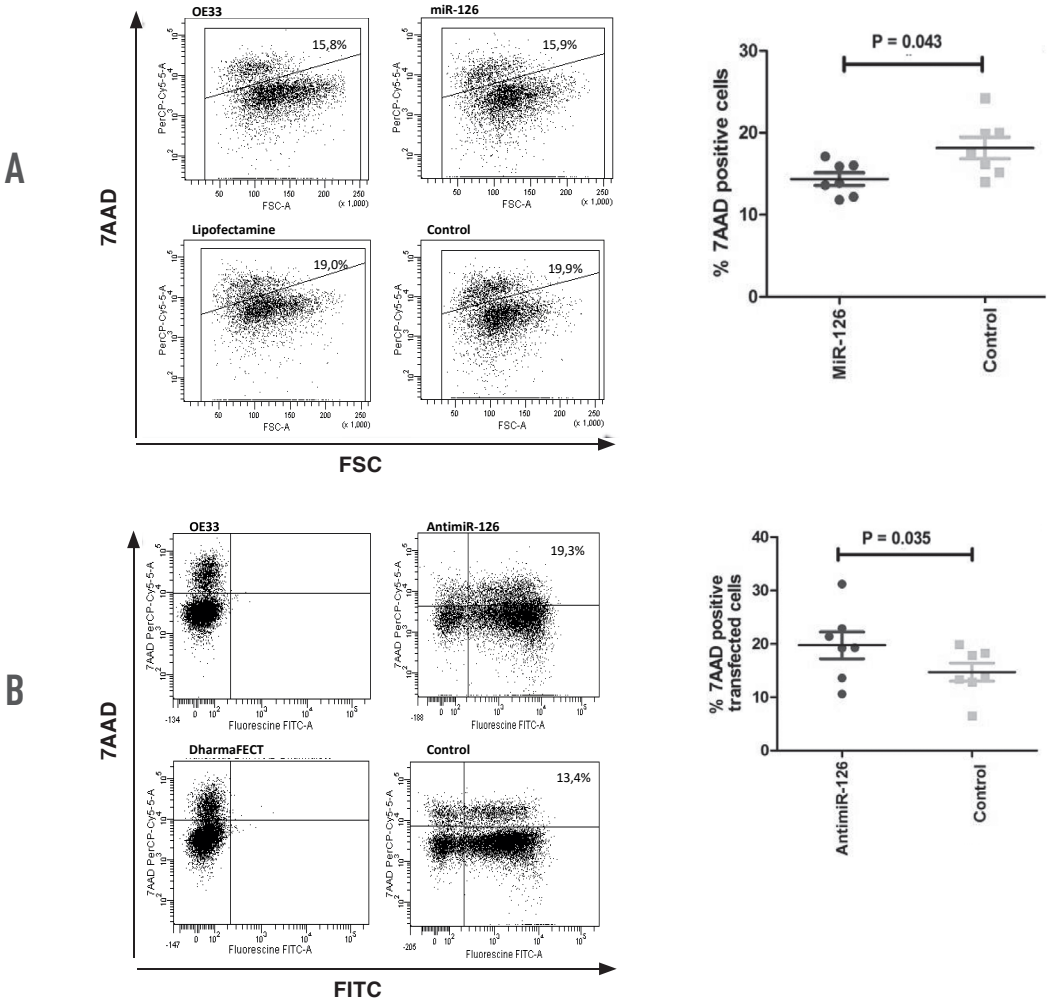


B



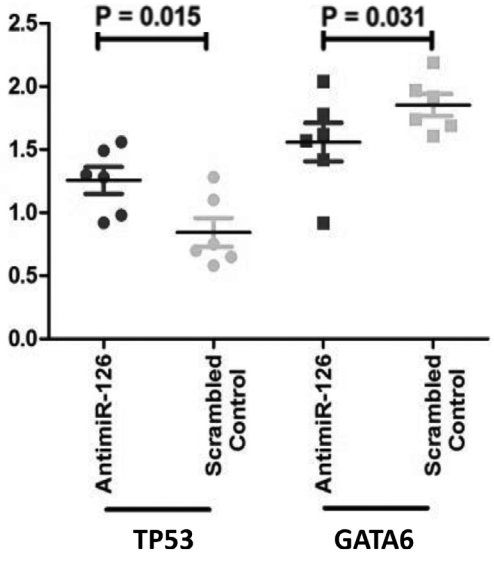
MiR-126 regulated MAP kinase and DNA damage related genes. Cancer pathway analysis was performed which includes 84 genes, representing nine major cancer pathways. Three independent experiments of paired OE33 cells either stably overexpressing miR-126 and an empty vector controls (CTR). A. Shown is fold change in normalised gene expression and the p-value of statistical analyses (paired T-test). B. MAP kinase MEK1 and DNA polymerase beta (POLB) were downregulated and telomeric repeat binding factor 1 (TERF1) significantly upregulated in samples overexpressing miR-126 compared to its empty vector control.

Figure 2



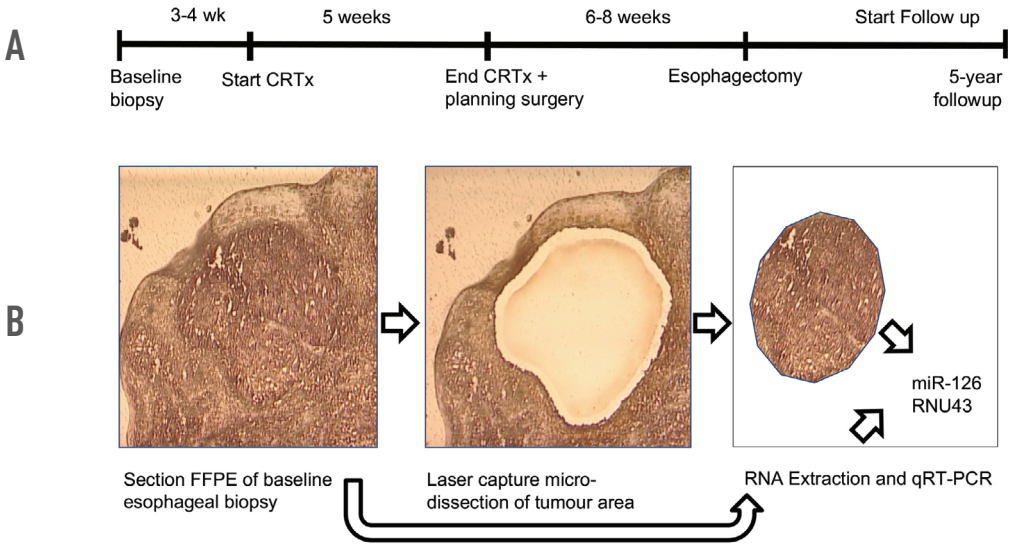
MiR-126 regulates cell death of OAC cells. A. Flowcytometric analysis of cell death in untreated OE33 cells, transfected OE33 cells overexpressing miR-126, OE33 cells treated with the transfection reagent Lipofectamin alone and transfected OE33 cells overexpressing an empty vector. Shown are representative plots with on the y-axis 7-AAD and on the x-axis the FSC (forward side scatter). The gated 7AAD positive cells represent the fraction of dead cells. Results of seven independent experiments show significantly less dead cells when overexpressing miR-126 compared to its empty vector. B. Shown are representative plots of untreated OE33 cells, OE33 cells transfected with antagonised miR-126 (antimiR-126), OE33 cells treated with the transfection reagent Darmafect alone and OE33 cells transfected with irrelevant antimiR control. On the y-axis 7-AAD and on the x-axis the FITC-channel is shown. Dead cells are represented in the double positive fraction (upper right corner). Results from seven independent experiments show significantly more dead cells when antagonising miR-126 compared to the control antimiR treated cells.

Figure 3



MiR-126 regulates genes relevant for apoptosis.
OE33 cells transfected with antagonised miR-126 (antimiR-126) were compared to transfected OE33 cells with irrelevant antimiR (scrambled) control. TP53 and GATA6 are plotted as fold changes. Results of six experiments are plotted showing antimiR-126 treatment significantly upregulates expression of TP53. A significantly lower expression of anti-apoptotic transcription factor GATA6 was seen in cells treated with antimiR-126.

Figure 4



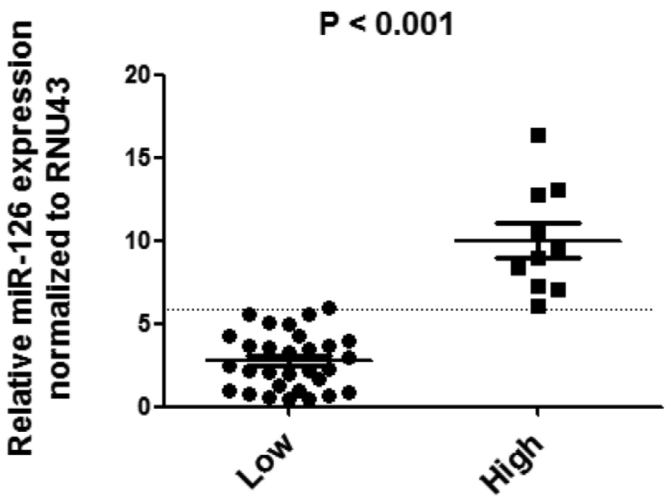
Evaluation of miR-126 levels in oesophageal biopsies of patients with OAC.

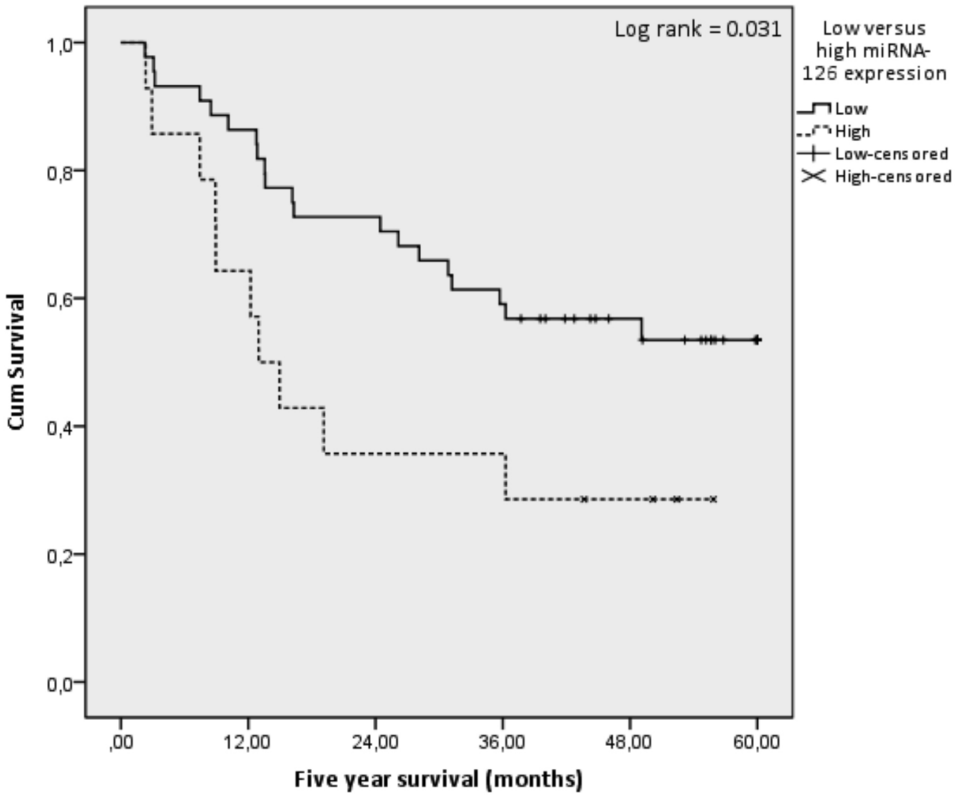
A. The time-line of treatment of patients with OAC and their follow up are shown. In total 58 patients were treated with combined neoadjuvant chemoradiotherapy and surgical resection and patient overall survival was monitored for 5 years post treatment.

B. Oesophageal biopsies taken at baseline containing both tumour and non-tumorous tissue including the squamous epithelium. Shown is a representative tissue section with HE-staining with in the middle a tumour-island (Left panel). Tumour-specific RNA was obtained by laser capture microdissection microscopy (Middle panel). Following microdissection, the tumour-piece was captured in a small tube ready for RNA isolation. Also RNA was extracted from the complete tissue section (Right panel).

C. Shown is the relative level of miR-126 normalised to RNU43 of 40 patients. Levels in tumour showed a wide variation (ranging from 0.4 to 16.4 normalised miR-126 values).

Figure 5


P < 0.001
Relative miR-126 expression normalized to RNU43
20
15
10
5
0
Low
High



Months	0	12	24	36	48	60
Low MiR126	44	38	32	26	25	24
High MiR126	14	9	5	5		

High tumour miR-126 levels are associated with poor survival in patients with OAC.
In 14 patients with high relative miR-126 levels in tumour (top25%), as compared to 44 patients with a median or low relative level of tumour miR-126, the average difference was 2.3-fold ($p<0.001$). B. Shown is five-year overall survival of the 58 patients treated with neoadjuvant chemoradiotherapy followed by surgery. High expression level of miR-126 in tumour tissue was significantly associated with poorer survival in patients with OAC. Cox regression analysis was used (Log rank $p=0.031$). The number of patients in each group at 12, 24, 36, 48 and 60 months post-treatments are shown at the bottom.

Chapter five

The prognostic value of preoperative serum gamma-glutamyltransferase in patients treated for oesophageal adenocarcinoma

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Submitted

Abstract

BACKGROUND

Recent studies have shown an association between serum biomarkers representing liver function and survival in patients with esophageal cancer. The aim of this study was to investigate whether serum liver function biomarkers are prognostic for overall survival (OS), disease-free survival (DFS) and pathological response to neoadjuvant chemoradiotherapy (nCRT) in patients with esophageal adenocarcinoma.

METHODS

Baseline and preoperative serum levels of the liver function biomarkers were determined in esophageal adenocarcinoma patients undergoing neoadjuvant chemoradiotherapy followed by esophagectomy. The association of serum gamma-glutamyltransferase (GGT), transaminases (ASAT and ALAT), albumin, total bilirubin and alkaline phosphatase with patient outcomes was assessed using logistic regression and Cox regression analysis.

RESULTS

Elevated baseline and preoperative levels of GGT were seen in 37 of 224 patients (16.5%) and 94 of 224 patients (42.0%), respectively. None of the serum liver function biomarkers were statistically significant associated with pathologically complete response in the resection specimen after multivariable analyses. Irrespective of therapy response, preoperative GGT was an independent predictor for 5-year DFS (HR 1.05 [95% CI 1.02–1.09], p=0.003) and 5-year OS (HR 1.04 [95% CI 1.00-1.07], p=0.032). An increase in GGT during nCRT had a negative impact on DFS (HR 1.05 [95% CI 1.01–1.09], p=0.024).

CONCLUSION

Preoperative GGT levels and increase of GGT during neoadjuvant therapy are associated with survival and could be a potential biomarker for survival in patients with esophageal adenocarcinoma.

Introduction

Esophageal cancer ranks as the 8th most common cancer and 6th most common cause of cancer deaths worldwide, affecting 456 000 patients annually [1]. Of both histologic subtypes, adenocarcinoma is predominant in Western countries and its incidence has been increasing rapidly over the past decades [2,3]. In many institutions patients with advanced locoregional disease are treated with neoadjuvant chemoradiotherapy (nCRT) followed by esophagectomy, leading to a 5-year survival of around 47% [4].
At present, the prognosis of patients with locally advanced disease who undergo neoadjuvant therapy and surgery with curative intent is based on robust histopathological parameters. The UICC-AJCC esophageal tumor-node-metastasis (TNM) staging system is used to estimate prognosis using pathological TNM stage and evidence of residual disease (R status) after surgery. Pretreatment or clinical TNM stage tends to be unreliable and variable with regard to prognosis. This emphasizes the need for more accurate prognostic factors before treatment to enable treatment stratification and to better inform patients about their prognosis [5].

Serum liver-derived proteins are frequently assessed in routine clinical practice. They are commonly elevated in patients with liver disease and may indicate liver injury [6]. Over the past years, several of these proteins have been studied in relation to treatment response and survival in esophageal cancer, including systemic inflammatory markers. Inflammation plays a crucial role in various stages of cancer development [7]. Presence of a tumor causes a systemic inflammatory response with a subsequent change in production of acute phase proteins such as C-reactive protein (CRP) and albumin by hepatocytes. CRP, albumin levels and the Glasgow Prognostic Score, an inflammation-based risk score, are associated with survival in esophageal cancer [8-13].
The most important serum biomarkers of liver injury are aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), of which the latter has been linked to response to neoadjuvant chemotherapy in esophageal carcinoma [14]. There is no evidence so far for a link between ASAT and response to neoadjuvant therapy. However, a relationship between an ALAT/ASAT ratio and 5-year OS was reported in patients with esophageal squamous cell carcinoma (ESCC) [15].
Gamma-glutamyltransferase (GGT) is an enzyme commonly used to identify liver dysfunction and high alcohol consumption [16]. Higher levels of GGT have been reported to be associated with overall survival (OS) as well as disease-free survival (DFS) in patients with ESCC (17). Recently, a second study was published that confirmed these findings [15]. Also, an association was found between postoperative complications and hyperbilirubinemia [18]. Similarly, alkaline phosphatase (ALP) levels were shown to be correlated with OS in ESCC [19].
Studies suggest a mechanistic role for serum liver-derived proteins in ESCC. The aim of the present study was to investigate whether the serum liver function biomarkers CRP, albumin, ASAT, ALAT, GGT, total bilirubin and ALP are associated with outcome in patients with esophageal adenocarcinoma. We hypothesized that elevated liver function biomarkers are associated with OS, DFS and pathological response to nCRT.

Materials and methods

PATIENTS

All patients with esophageal adenocarcinoma that received nCRT followed by esophagectomy at the Erasmus University Medical Center Rotterdam, the Netherlands between April 2001 and May 2016 were included [4,20,21]. Excluded were patients with squamous cell carcinoma, patients with an irresectable tumor and patients with distant metastasis. The ethical committee of the Erasmus MC provided ethical approval prior to commencement of the study.

TREATMENT

Neoadjuvant chemoradiation consisted of 5 cycles of chemotherapy with paclitaxel and carboplatin and concurrent radiotherapy with a total dose of 41.4 Gray given in 23 fractions (4, 20, 21). A transthoracic or transhiatal approach was used for esophagectomy depending on the patient's condition, location of the tumor and preference of the surgeon [22,23].

STAGING

The 7th edition of the TNM Classification of Malignant Tumors was used to assess clinical and pathological TNM stage [24]. Patient work-up consisted of physical examination and routine hematological and biochemical tests, along with upper gastrointestinal endoscopy with biopsies and endoscopic ultrasonography with fine-needle aspiration (FNA) of suspected lymph nodes on indication. In addition, a computed tomography (CT) scan of neck, chest and abdomen was performed. Positron emission tomography was used in selected patients but was not incorporated in the standard examination. Ultrasonography of the neck with FNA was performed when indicated.

DATA COLLECTION

The study was designed as a retrospective cohort study. For all patients, data on baseline patient demographics and tumor characteristics as well as the postoperative course were obtained from a prospectively recorded institutional database. The Charlson Comorbidity Index (CCI) was used to assess comorbidities [25]. Percentage of weight loss was calculated by dividing weight loss at diagnosis by the premorbid weight. Dysphagia was scored according to the following classification: 0 = no complaints, 1 = sporadic obstruction of solid foods, 2 = patient able to eat semisolid food, 3 = patient able to eat liquid food, 4 = patient not able to swallow saliva. Nutritional support was defined by the use of nutritional drinks and/or enteral feeding.

Baseline and preoperative laboratory values of CRP, albumin, ASAT, ALAT, GGT, total bilirubin and ALP were obtained from routine biochemical tests stored in the electronic patient database. Liver function tests were determined at the preoperative screenings unit of the department of Anesthesiology. These were measured in serum using a Roche Modular or Roche Cobas

8000 (Roche, Almere, The Netherlands). In a small number of patients (n = 30), serum had been stored and baseline CRP, albumin, ASAT and ALAT levels were determined in retrospect using the standard procedure. If multiple baseline values were present, for each liver parameter the value closest to start of chemoradiotherapy was used for the analyses. This could be a value derived from the first visit to the outpatient clinic or the data was extracted from the referral letter in case the diagnosis was made at another hospital. For preoperative values, the last available value prior to the date of surgery was used in the time frame between end of nCRT and date of surgery. In some patients, neither or only one of both values was available. Any decrease or increase (trends) in liver function biomarkers during nCRT was noted by subtracting baseline from preoperative values. Levels of liver function biomarkers were interpreted using the reference values for adults in the Erasmus MC.

SURVIVAL AND RESPONSE TO NCRT

The primary outcomes of the study were pathological response to nCRT and survival. OS was calculated from date of surgery until date of death or end of follow-up (May 26th 2016). DFS was defined as the time from date of surgery until date of first recurrence or end of follow-up. In the first 5 years after surgery, patients visited the outpatient clinic every three months the first year, six-monthly for year two and yearly thereafter. Radiological examinations were performed only when there was a suspicion for recurrent disease. Correlation between the liver function biomarkers and long-term (5-year) survival was assessed.
Response to nCRT was assessed in the resection specimen by an experienced pathologist using the Mandard score (tumor regression grade = TRG) [26]. A modified score from 1 to 4 was used to assess TRG, with TRG 1 = no vital residual tumor cells at the site of the primary tumor nor in the resected lymph nodes (pathologically complete response; pCR), TRG 2 = less than 10% vital tumor cells remaining in tumor, lymph nodes or both, TRG 3 = 10-50% vital remaining tumor cells, TRG 4 = more than 50% vital tumor cells remaining [27].

STATISTICAL ANALYSIS

Data were analyzed using SPSS version 24.0 (SPSS, Chicago, Illinois, USA). In all analyses, a two-sided p-value of <0.05 was used for statistical significance. Multiple imputation by fully conditional specification (FCS) with 20 imputations was used to take into account missing data on the liver function biomarkers, patient demographics and tumor characteristics when executing logistic and Cox regression analyses. The remaining analyses were done using the original database. Baseline albumin, ASAT, ALAT, GGT, total bilirubin and ALP had 19.2, 6.3, 5.8, 8.9, 11.2 and 8.0 percent missing values respectively. Baseline CRP had over 50% missing values as it was not routinely assessed and was therefore not included in further analyses. Preoperative CRP, albumin, ASAT, ALAT, GGT, total bilirubin and ALP had 75.0, 52.2, 4.9, 4.9, 12.5, 11.2 and 7.1 percent missing values respectively. Preoperative CRP and albumin were deemed to have too many missing values for accurate imputation and also excluded from further analyses. Group differences in liver function biomarker levels were evaluated by Mann-Whitney U or Kruskal-Wallis H test. Correlations of continuous variables were analyzed using Spearmans rank correlation coefficient. Significance of trends over time was evaluated using the Wilcoxon test. All figures were obtained using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA).

The relationship between the liver function biomarkers and pathological response to nCRT was analyzed using Logistic regression analysis. Odds ratios (OR) with 95% confidence interval (CI) were calculated. The correlation of the liver biomarkers with OS and DFS was analyzed using Cox regression analysis as well as the Kaplan-Meier method with log-rank test to evaluate significance. Surviving patients were censored on the last day of follow-up, May 26th 2016. Data are expressed as hazard ratios (HR) with 95% CI. The liver function biomarkers along with prognostic factors were tested for significance in univariable analysis, and variables with a p-value <0.10 in univariable analysis were added to a multivariable model. Potential non-linear correlations of the markers with outcome were examined using Martingale or Pearson residual plots with a Loess fit line and transformed when indicated.

Results

A total number of 224 patients diagnosed between December 2000 and January 2016 were enrolled in the study. Baseline characteristics including the number of missing values are shown in Table 1. Two (0.9%) patients had decreased albumin levels. Elevated baseline ASAT and ALAT levels were present in 19 (8.5%) and 21 (9.4%) patients. Baseline GGT, total bilirubin and ALP levels were elevated in 37 (16.5%), 9 (4.0%) and 11 (4.9%) patients, respectively. Two (0.9%) patients had a history of hepatitis. There were 20 (8.9%) patients with type 2 diabetes.

ASSOCIATION BETWEEN BASELINE LIVER FUNCTION BIOMARKERS AND CLINICOPATHOLOGICAL PARAMETERS

The association between baseline liver function biomarkers and clinicopathological parameters is shown in Supplementary Table S1. While albumin and ASAT were significantly associated with cT stage (p=0.048 and p=0.027, respectively), none of the markers were associated with cN stage. ASAT was the only marker that was significantly higher in patients with increasing self-reported alcohol intake (p=0.046, Supplementary Figure S1). GGT levels were significantly higher in males. In addition, albumin and total bilirubin were correlated with tumor length (Spearmans correlation coefficient (Rs) = -0.259 and -0.188; p<0.001 and 0.008 resp.) while albumin correlated significantly with age (Rs = -0.268; p<0.001). Also, a significant correlation between ASAT (Rs = 0.18; p=0.009), ALAT (Rs = 0.225; p=0.001), GGT (Rs = 0.183; p=0.009) and BMI was found. Furthermore, a correlation between baseline albumin (Rs = -0.288; p<0.001), ASAT (Rs = -0.255; p<0.001), ALAT (Rs = -0.146; p=0.034), and percentage of weight loss was seen.

LIVER FUNCTION BIOMARKERS AND RESPONSE TO CHEMORADIOTHERAPY

Pathology of the resection specimens is shown in Table 2. One patient had an ESCC after examination of the resection specimen. Forty-four patients (19.6%) had a pCR (TRG1). Higher baseline levels of ASAT were found in patients with pCR compared to patients with residual tumor (median 24 vs. 22, p=0.040, Figure 1). No statistically significant differences for other biomarkers were found between patients with pCR and residual tumor. ASAT was not an independent predictor of pCR (Supplementary Table S2). Individual preoperative values and trends in liver function biomarkers were not predictive of response to nCRT.

CHANGES IN LIVER FUNCTION BIOMARKERS FROM BASELINE TO TIME OF SURGERY

Figure 2 shows the changes in liver function biomarkers between baseline and presurgery for the individual patients. In Supplementary Figure S2 these trends are shown separated into patients with pCR and patients with residual tumor. A change in GGT levels during therapy was predictive of 5-year DFS (per 10 units, HR 1.04 [95% CI 1.00–1.08], p=0.042), an increase being predictive of worse survival. Likewise, changes in ALP were predictive of 5-year DFS (per 10 units, HR 1.12 [95% CI 1.01–1.23], p=0.030), an increase during therapy being unfavorable (Table 3). When analyzed separately in multivariable analyses, only GGT had a significant impact on DFS (per 10

units, HR 1.05 [95% CI 1.01–1.09], p=0.024) whereas ALP and total bilirubin did not. Trends in liver function biomarkers were not predictive of 5-year OS (Supplementary Table S3).

RELATIONSHIP OF BASELINE LIVER FUNCTION BIOMARKERS TO SURVIVAL

Five-year OS and DFS in this cohort were 56.3% and 62.3% respectively. Median OS was 55.7 months (IQR [16.2 to 155.1]). Median DFS was 100.4 months (IQR [11.3 to 140.5]). Of the 107 patients that underwent surgery and did not survive, 83 (77.6%) died from recurrence and 24 (22.4%) from other causes (6 from a second primary tumor, 5 from respiratory insufficiency, 2 from (sepsis with) multi-organ failure, 2 after reconstructive surgery for a tracheoesophageal fistula, 2 from a (thromboembolic) stroke, 1 from cardiac asystole, 1 from bowel ischemia and 5 from an unknown cause). None of the liver function biomarkers were associated with 5-year OS and DFS (Supplementary Table S4). In addition, alcohol use was not found to be a significant predictor of OS nor of DFS upon univariable analysis (Data not shown).

PREOPERATIVE LEVELS OF LIVER FUNCTION BIOMARKERS AND SURVIVAL

Preoperative ASAT, ALAT, GGT, total bilirubin and ALP had 11 (4.9%), 11 (4.9%), 28 (12.5%), 25 (11.2%) and 16 (7.1%) missing values respectively. Median values of preoperative ASAT and ALAT were 28 (range 12 to 73) and 27 (range 7 to 156) U/L, respectively. Median GGT and total bilirubin were 51 (range 9 to 309) U/L and 8 (range 2 to 35) µmol/L respectively. Median ALP was 81 (range 23 to 210) U/L. Preoperative values for ASAT and ALAT were elevated in 42 (18.8%) and 32 (14.3%) patients respectively. For GGT, total bilirubin and ALP, 94 (42.0%), 6 (2.7%) and 31 (13.8%) patients had elevated levels, respectively. Similar to baseline GGT levels, preoperative levels of GGT were not significantly higher in patients with more alcohol intake at baseline (medians of 45 vs. 57.5 vs. 49 resp. with increasing alcohol use; p=0.29 on Kruskall Wallis test). Median time from date of measurement until date of surgery was 1 day (IQR [1 to 3]), while median time from end of nCRT until date of measurement was 47 days (IQR [31.3 to 64.8]). In Figure 3 5-year DFS according to elevation of GGT levels is shown.

Preoperative GGT levels was an independent predictor of 5-year DFS (per 10 units, HR 1.05 [95% CI 1.02–1.09], p=0.003, Table 4). Preoperative GGT levels showed a trend towards significance in being predictive of 5-year OS in univariable analysis, with a higher preoperative level being predictive of worse survival (Table 4). When corrected for tumor length, cT stage, cN stage and percentage of weight loss, GGT was also an independent predictor of OS (per 10 units, HR 1.04 [95% CI 1.00–1.07], p=0.032). However, despite its statistical significance, only a small effect of GGT on survival was seen. Individual preoperative values of ASAT, ALAT and total bilirubin were not predictive of DFS nor of OS.

Discussion

The current study aimed to establish the role of serum liver function biomarkers CRP, albumin, ASAT, ALAT, GGT, total bilirubin and ALP in the prognostication of patients with esophageal adenocarcinoma. Our study found that higher baseline serum ASAT levels were associated with a higher rate of pCR, but ASAT was not an independent predictor of pathological response to nCRT. More importantly, we found that preoperative GGT was an independent predictor of DFS and OS. In addition, an increase in GGT during chemoradiotherapy had a negative impact on DFS.

Unfortunately, it was not possible to assess the effect of CRP on outcome as it was not regularly assessed in our cohort. In addition, we were not able to reproduce the correlation of albumin with survival and response to nCRT in the current study but this may be explained by the fact that some previous studies used cut-off points in their analyses.
The correlation between ASAT and response to nCRT is surprising as elevated ASAT is commonly considered an indicator of liver injury. However, our study population contained only patients with baseline ASAT levels of no more than twice the upper limit, suggesting a different pathophysiological mechanism within this range. It must however be interpreted with caution as no independent effect of ASAT on pCR was found. No link between ALAT and response to nCRT could be established in the current study.

The correlation of preoperative GGT levels with survival in esophageal cancer patients has been reported previously [15-17]. The correlation of high levels with survival has also been demonstrated in various other malignancies including endometrial, cervical, colon, hepatocellular and renal cell carcinoma [28-32]. We were able to confirm these findings in the present study. Interestingly, we did not find a correlation of GGT levels with pCR whereas pCR is known to be an important determinant of disease recurrence and overall survival [33,34]. Our results therefore suggest a relationship of GGT with survival independent of response to nCRT. In the present study less than half of patients had an elevated preoperative GGT level which suggests a detrimental effect of increasing GGT levels also within the (upper) normal range.

GGT is a cell membrane-bound enzyme involved in glutathione metabolism. Glutathione has antioxidant properties and protects cells against oxidants that are produced during cell metabolism (16). Therefore, oxidative stress as present in carcinogenesis causes an increase in GGT and glutathione. The enzyme is recognized as a modulator of antioxidant and antitoxic defences, and of the cellular proliferative and apoptotic balance. Increased levels of GGT have been observed in various neoplasms [35]. Previous literature has suggested that GGT may play a role in resistance to anticancer drugs. In addition, there is evidence for its role in tumor invasion and progression and its expression has been shown to enable faster tumor growth in vivo [35,38]. These findings may provide an explanation for the detrimental effects of higher GGT levels on survival. It is well known that GGT levels are also modulated by lifestyle factors of which alcohol use is the most significant. Thus, alcohol use could be another explanatory mechanism behind the detrimental effects of higher GGT levels on survival. However, the present study did not find a correlation between levels of alcohol use and increasing GGT levels. In addition, alcohol use was not found to be a prognosticator for survival.

While baseline GGT was not predictive of survival, preoperative GGT and its course over time were. This suggests a change in the clinical impact of GGT during treatment, be it either causal of worse survival or as an epiphenomenon indicative of an underlying process taking place that modifies survival.

Previous literature on the relationship between GGT and survival in esophageal cancer focused solely on patients with ESCC. In addition, previous studies included patients undergoing different treatment regimens. Our study included only patients that received nCRT followed by esophagectomy, which allowed us to validate preoperative GGT as a biomarker for survival in a large independent cohort of patients with esophageal adenocarcinoma undergoing a consistent treatment regimen. These findings substantiate the potential use of preoperative GGT as a universal marker for survival in patients with esophageal carcinoma.
A limitation of the current study is the retrospective design resulting in some missing data. However, a solid model was applied, which allowed for accurate imputation of missing values. In addition, the majority of data was prospectively collected and the number of missing values in liver function biomarkers and clinical characteristics was relatively low. For survival, relatively small correlations of GGT were seen compared to well-established prediction factors such as the TNM staging system, requiring more research into the clinical applicability of our findings.
In conclusion, this study offers insight in the practical use and added value of liver function biomarkers in the prognostication of esophageal adenocarcinoma. Preoperative GGT levels and the changes of GGT during neoadjuvant chemoradiotherapy can be easily determined at low costs which make it an attractive biomarker.

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Chapter five

tables and figures

Table 1

Characteristics and laboratory values of 224 patients with esophageal adenocarcinoma

Characteristic	All patients (n = 224)
Age (years)	
Median (range)[1]	63 (18 – 84)
Gender, no. (%)	
Male	198 (88.4)
Female	26 (11.6)
Differentiation grade, no. (%)	
Good	6 (2.7)
Moderate	116 (51.8)
Poor	71 (31.7)
Unknown	31 (13.8)
Tumor length (cm)	
Median (range)[1]	5 (1 – 14)
Tumor location, no. (%)	
Mid-esophagus	10 (4.5)
Distal esophagus	165 (73.7)
Gastroesophageal junction	49 (21.9)
Clinical tumor (cT) stage, no. (%)	
cT1	9 (4.0)
cT2	37 (16.5)
cT3	165 (73.7)
cT4a	9 (4.0)
Could not be determined[2]	4 (1.8)
Clinical nodal (cN) stage, no. (%)	
cN0	75 (33.5)
cN1	76 (33.9)
cN2	62 (27.7)
cN3	9 (4.0)
Could not be determined[2]	2 (0.9)
Charlson comorbidity index (CCI), no. (%)	
No comorbidity	144 (64.3)
One or more comorbidities	80 (35.7)
Body Mass Index (BMI, kg/m^2)	
Median (range)[1]	26.6 (18 – 39.8)
Percentage weight loss	
Median (range)[1]	3.7 (0 – 31.6)
Dysphagia grade, no. (%)	
0	36 (16.1)
1	126 (56.3)
2	21 (9.4)
3	32 (14.3)
4	2 (0.9)
Unknown	7 (3.1)
Nutrition support[3], no. (%)	
None	150 (67.0)
Any	72 (32.1)
Unknown	2 (0.9)
Alcohol use, no. (%)	
None	43 (19.2)
Less or equal to 2 units a day	93 (41.5)
More than 2 units a day	86 (38.4)
Unknown	2 (0.9)
Smoking, no. (%)	
Non-smoking or quitted	154 (68.8)
Less than ½ pack a day	30 (13.4)
More than ½ pack a day	39 (17.4)
Baseline albumin (g/L)	
Median (range)[4] **(35-50)**[5]	44 (30.9-51)
Baseline ASAT (U/L)	
Median (range)[4] **(M: <35, F: <31)**[5]	23 (10-55)
Baseline ALAT (U/L)	
Median (range)[4] **(M: <45, F: <34)**[5]	22 (6-86)
Baseline GGT (U/L)	
Median (range)[4] **(M: <55, F: <38)**[5]	29 (10-392)
Baseline total bilirubin (µmol/L)	
Median (range)[4] **(<17)**[5]	8 (2-26)
Baseline ALP (U/L)	73 (21-168)
Median (range)[4] **(M: <115, F: <98)**[5]	

[1] Age, Body Mass Index and percentage weight loss had no missing values. Tumor length had 4 (1.8%) missing values at baseline

[2] cT stage or cN stage could not be determined using endoscopic ultrasound

[3] Nutrition support comprises of nutritional drinks or enteral feeding, or both

[4] 43 (19.2%), 14 (6.3%), 13 (5.8%), 20 (8.9%), 25 (11.2%) and 18 (8.0%) missing values respectively at baseline

[5] Reference values for adults as employed by the Erasmus MC. M: male, F: female

Table 2

Pathological assessment of the resection specimen of 224 patients with esophageal cancer

Characteristic	All patients (n = 224)
Histology, no. (%)	
Adenocarcinoma	223 (99.6)
Squamous cell carcinoma	1 (0.4)
Differentiation grade, no. (%)	
ypGx[1]	68 (30.3)
ypG1	3 (1.3)
ypG2	83 (37.1)
ypG3	69 (30.8)
ypG4	1 (0.4)
Pathological tumor (ypT) stage, no. (%)	
ypT0	50 (22.3)
ypT1	40 (17.9)
ypT2	45 (20.1)
ypT3	88 (39.3)
ypT4a	1 (0.4)
Radicality, no. (%)	
ypR0	209 (93.3)
ypR1	15 (6.7)
Pathological nodal (ypN) stage, no. (%)	
ypN0	140 (62.5)
ypN1	64 (28.6)
ypN2	13 (5.8)
ypN3	7 (3.1)
Pathological metastasis (ypM) stage, no. (%)	
ypM0	222 (99.1)
ypM1[2]	2 (0.9)
Resected lymph nodes	
Median (range)	16 (0 – 76)
Tumor regression grade (TRG), no. (%)	
TRG 1	44 (19.6)
TRG 2	68 (30.4)
TRG 3	69 (30.8)
TRG 4	42 (18.8)
Unknown	1 (0.4)

[1] Differentiation grade could not be determined. This includes patients with a pathologically complete response in which no tumor is left in the resection specimen

[2] Patients in which metastasis of the liver was discovered peroperatively but still underwent esophagectomy

Table 3

Univariable and multivariable analysis of trends in liver function biomarkers and 5-year disease-free survival

	Univariable analysis			Multivariable analysis[3]		
Variable	HR	95% CI	P value	Adjusted HR	95% CI	P value
Age per decade	1.01	(0.81-1.26)	0.95			
Sex						
Male	Ref					
Female	0.77	(0.53-1.11)	0.47			
Differentiation grade						
Good or moderate	Ref					
Poor	1.19	(0.76-1.87)	0.45			
Tumor length	1.10	(1.02-1.19)	**0.014**	1.04	(0.95-1.14)	0.41
Tumor location						
Mid-esophagus	Ref					
Distal esophagus	0.54	(0.23-1.26)	0.15			
Gastroesophageal junction	0.86	(0.35-2.13)	0.75			
Clinical tumor (cT) stage						
1 or 2	Ref			Ref		
3 or 4	2.64	(1.36-5.13)	**0.004**	2.19	(1.08-4.41)	**0.029**
Clinical nodal (cN) stage						
0	Ref			Ref		
1	1.90	(1.08-3.36)	**0.027**	1.60	(0.89-2.88)	0.12
2 or 3	2.33	(1.33-4.09)	**0.003**	1.50	(0.81-2.77)	0.20
CCI						
None	Ref					
One or more comorbidities	1.19	(0.77-1.84)	0.44			
Percentage weight loss	1.07	(1.03-1.11)	**<0.001**	1.05	(1.01-1.09)	**0.008**
Liver function biomarker[1,2]						
Increase in ASAT	1.12	(0.93-1.35)	0.24			
Increase in ALAT	1.07	(0.96-1.19)	0.23			
Increase in GGT	1.04	(1.00-1.08)	**0.042**	1.05	(1.01-1.09)	**0.024**
Increase in total bilirubin	0.59	(0.33-1.07)	0.082	0.59	(0.32-1.10)	0.096
Increase in alkaline phosphatase	1.12	(1.01-1.23)	**0.030**	1.08	(0.97-1.20)	0.16

[1] Per 10 units increase in liver function biomarker

[2] Trends in liver function biomarkers were calculated by subtracting baseline values from preoperative values

[3] Multivariable analyses for clinicopathological parameters are shown with only GGT added to the multivariable model, being the only significant marker in multivariable analysis

Table 4

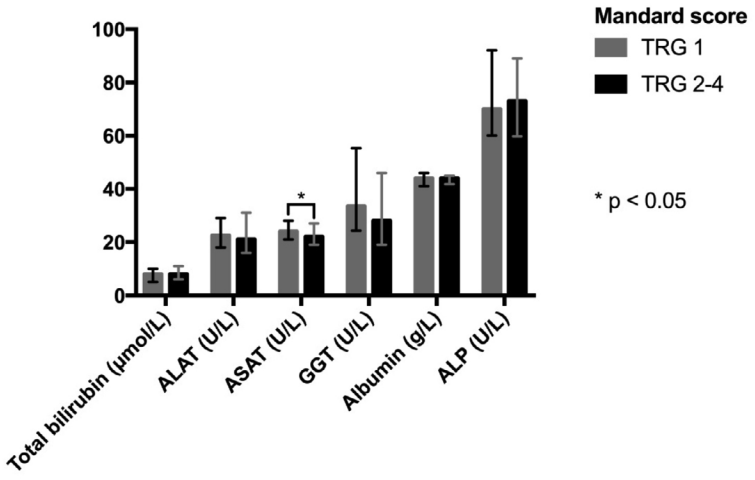
Univariable and multivariable analysis on preoperative levels of the liver function biomarkers and five-year disease-free survival and 5-year overall survival

	5-year DFS						5-year OS					
	Univariable analysis			Multivariable analysis			Univariable analysis			Multivariable analysis		
Variable	HR	95% CI	p value	Adjusted HR	95% CI	p value	HR	95% CI	p value	Adjusted HR	95% CI	p value
Age per decade	1.01	(0.81-1.26)	0.95				1.19	(0.96-1.48)	0.12			
Sex												
Male	Ref						Ref					
Female	0.77	(0.53-1.11)	0.47				1.04	(0.57-1.90)	0.91			
Differentiation grade												
Good or moderate	Ref						Ref					
Poor	1.19	(0.76-1.87)	0.45				1.02	(0.67-1.56)	0.93			
Tumor length	1.10	(1.02-1.19)	**0.014**	1.04	(0.95-1.15)	0.37	1.08	(1.00-1.16)	**0.047**	1.03	(0.94-1.13)	0.53
Tumor location												
Mid-esophagus	Ref						Ref					
Distal esophagus	0.54	(0.23-1.26)	0.15				0.66	(0.29-1.52)	0.33			
Gastroesophageal junction	0.86	(0.35-2.13)	0.75				0.94	(0.38-2.31)	0.89			
Clinical tumor (cT) stage												
1 or 2	Ref			Ref			Ref			Ref		
3 or 4	2.64	(1.36-5.13)	**0.004**	2.29	(1.13-4.63)	**0.021**	2.29	(1.28-4.13)	**0.006**	2.04	(1.09-3.82)	**0.026**
Clinical nodal (cN) stage												
0	Ref			Ref			Ref			Ref		
1	1.90	(1.08-3.36)	**0.027**	1.54	(0.85-2.79)	0.15	1.36	(0.82-2.26)	0.24	1.15	(0.68-1.95)	0.61
2 or 3	(1.33-4.09)		**0.003**	1.44	(0.78-2.67)	0.25	(1.08-2.92)		**0.024**	1.20	(0.69-2.08)	0.52
	2.33						1.78					
CCI												
None	Ref						Ref					
One or more comorbidities	1.19	(0.77-1.84)	0.44				1.33	(0.88-2.00)	0.18			
Percentage weight loss	1.07	(1.03-1.11)	**<0.001**	1.05	(1.01-1.09)	**0.009**	1.06	(1.02-1.10)	**0.001**	1.05	(1.01-1.08)	**0.021**
Liver function biomarker[1]												
ASAT	0.98	(0.78-1.23)	0.85				1.00	(0.81-1.23)	0.97			
ALAT	1.04	(0.93-1.17)	0.50				1.02	(0.91-1.14)	0.73			
GGT	1.04	(1.01-1.08)	**0.010**	1.05	(1.02-1.09)	**0.003**	1.03	(1.00-1.06)	0.073	1.04	(1.00-1.07)	**0.032**
Total bilirubin	1.04	(0.33-1.22)	0.17				1.04	(0.48-1.47)	0.54			
Alkaline phosphatase	1.04	(0.97-1.14)	0.25				1.03	(0.94.1.10)	0.68			
	0.63						0.84					
	1.05						1.02					

[1] Per 10 units increase in liver function biomarker

Figure 1

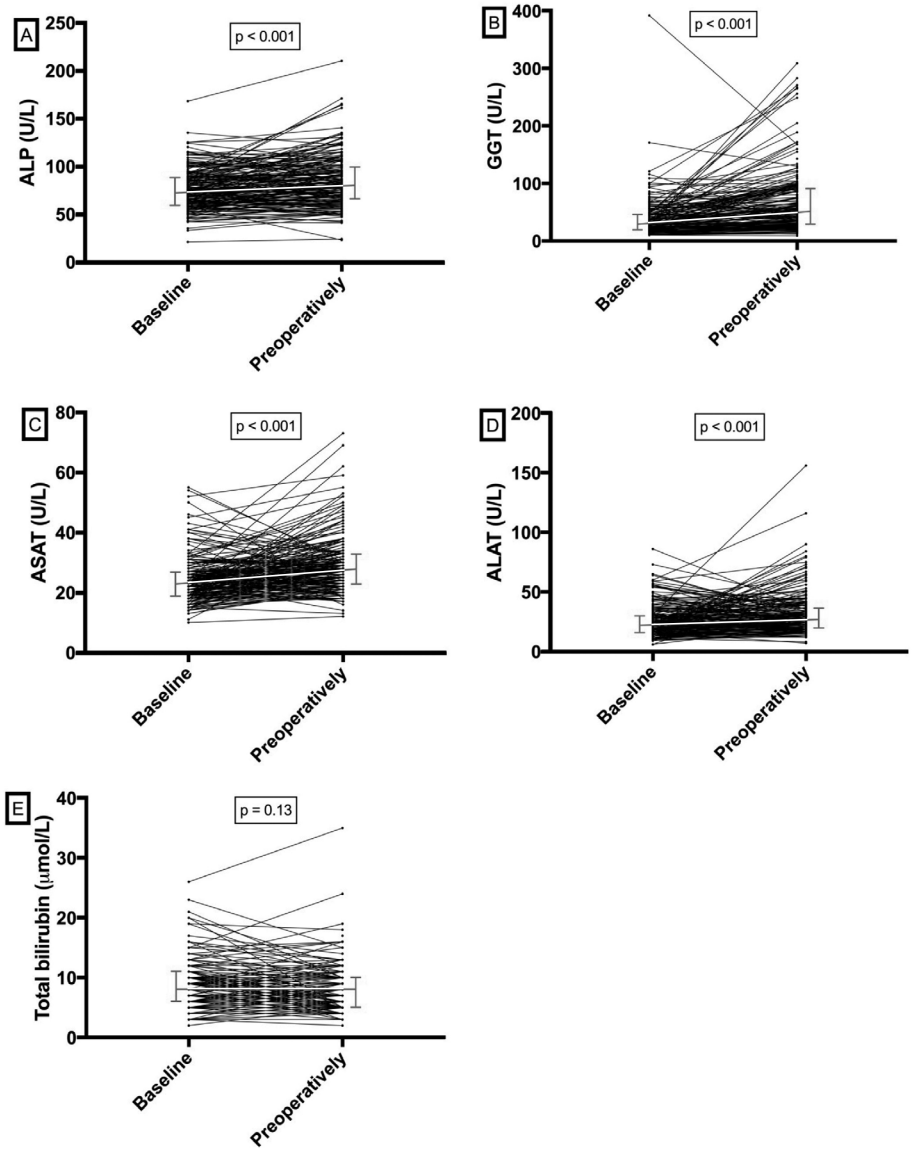
Univariable and multivariable analysis of trends in liver function biomarkers and 5-year disease-free survival



Median baseline values of liver function biomarkers for patients with a pathologically complete response (TRG 1) and incomplete response (TRG 2-4). Displayed are the median values with interquartile range. Only baseline ASAT levels were significantly higher in complete responders compared to patients with incomplete response (medians of 24 vs. 22, p=0.040 on Mann-Whitney test).

Figure 2

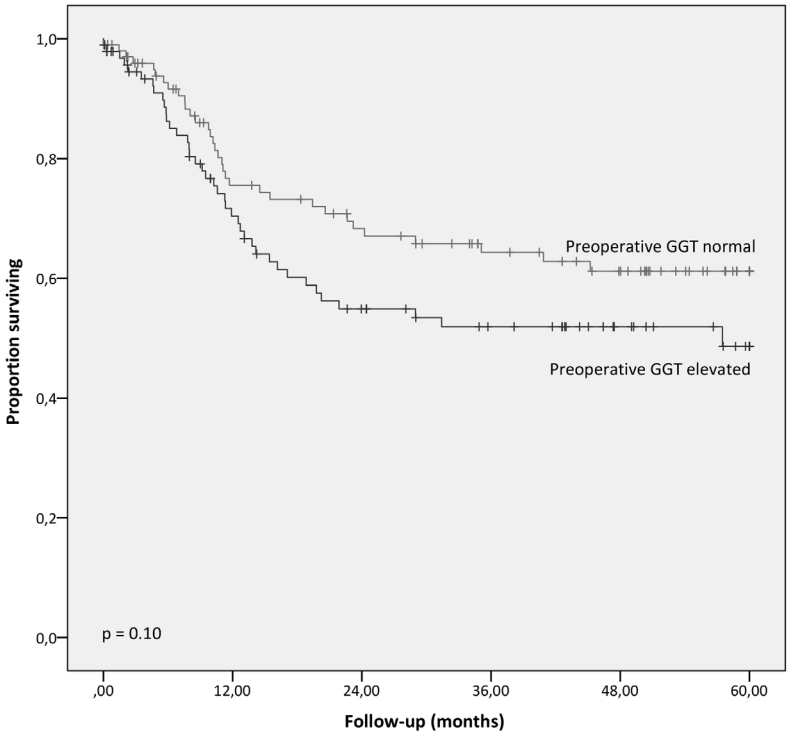
Univariable and multivariable analysis on preoperative levels of the liver function biomarkers and five-year disease-free survival and 5-year overall survival



Changes in liver function biomarkers. Plots A-E show lines of individual patients with their baseline and preoperative levels of serum liver function parameters. Median values with interquartile ranges at baseline and preoperatively are displayed in red. Median increase in ALP from baseline was 7 U/L (interquartile range (IQR) [-3 to 19]; p<0.001 on Wilcoxon test). GGT and ASAT increased with medians of 13 U/L (IQR [3 to 43]; p<0.001) and 5 U/L (IQR [-1 to 10]; p<0.001) respectively. Median positive change in ALAT and total bilirubin were 4 U/L (IQR [-2.5 to 11.5]; p<0.001) and 0 µmol/L (IQR [-2 to 1]; p=0.13).

Figure 3

Univariable and multivariable analysis of trends in liver function biomarkers and 5-year disease-free survival



Number of patients at risk						
Normal GGT	102	65	55	44	36	17
Elevated GGT	94	56	40	32	21	11
Total	196	121	95	76	57	28

5-year disease-free survival according to elevation of GGT levels. There was no statistically significant difference between the two groups (logrank test p = 0.10).

PART II

Multimodality Treatment

Chapter six

Nomogram for predicting response to neoadjuvant chemoradiotherapy

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Abstract

BACKGROUND

A pathologically complete response (pCR) to neoadjuvant chemoradiotherapy (nCRT) is seen in 30% of the patients with oesophageal cancer. The aim is to identify patient and tumour characteristics associated with a pCR and to develop a nomogram for the prediction of pCR.

PATIENTS AND METHODS

Patients who underwent nCRT followed by surgery were identified and response to nCRT was assessed according to a modified Mandard classification in the resection specimen. A model was developed with age, gender, histology and location of the tumour, differentiation grade, alcohol use, smoking, percentage weight loss, Charlson Comorbidity Index (CCI), cT-stage and cN-stage as potential predictors for pCR. Probability of pCR was studied via logistic regression. Performance of the prediction nomogram was quantified using the concordance statistic (c-statistic) and corrected for optimism.

RESULTS

A total of 381 patients were included. After surgery, 27.6% of the tumours showed a pCR. Female sex, squamous cell histology, poor differentiation grade, and low cT-stage were predictive for a pCR with a c-statistic of 0.64 (corrected for optimism).

CONCLUSION

A nomogram for the prediction of pathologically complete response after neoadjuvant chemoradiotherapy was developed, with a reasonable predictive power. This nomogram needs external validation before it can be used for individualized clinical decision-making.

Introduction

Oesophageal cancer is an aggressive disease, with rising incidences in the United States and Western Europe [1,2]. It is often diagnosed at an advanced stage; hence less than half of the patients are eligible for potentially curative treatment. Neoadjuvant chemoradiotherapy (nCRT) followed by surgery is considered standard treatment for locoregional disease (cT1N1 and cT2-T4a, N0-N3, M0). The purpose of this multimodality approach is to downstage the primary tumour and regional lymph nodes in order to facilitate a radical resection and to eradicate micrometastases. Recently, the Dutch CROSS trial showed that patients who underwent nCRT followed by surgery had a better survival with reduced locoregional and distant recurrences as compared to surgery alone [3-6].

The extent of tumour regression in the resection specimen, as assessed by the pathologist, in the primary lesion and removed lymph nodes is associated with survival after oesophagectomy [7,8]. Approximately 30% of patients receiving nCRT followed by surgery have a pathologically complete response (pCR; i.e. no vital tumour cells in the resection specimen) [6]. Patients with pCR have a better overall survival compared to patients with residual disease in the resection specimen [4,9-11]. Patients without substantial pathological response to nCRT do not seem to benefit from nCRT but are exposed to toxicity of nCRT. Furthermore, curative surgery is delayed [12].

Identification of patients who will benefit most from nCRT has been the subject of several studies. If we were able to accurately predict pCR in individual patients, these patients might be candidates to postpone or even omit surgical resection. Biomarker and tumour genetic profiles have been investigated, but they are complex to use and none have been properly validated. Functional imaging, including positron emission tomography is moderately able to identify responders early during neoadjuvant chemotherapy, but is less accurate for the early assessment of tumour response to nCRT [13,14].

The aim of this retrospective cohort study was to identify patient and tumour characteristics that are associated with pathologically complete response after nCRT. Secondly, we sought to develop a nomogram that is able to predict pathologically complete response in an attempt to identify potential patients in whom subsequent resection might be postponed or even omitted.

Patient and methods

PATIENTS

A total of 381 patients with histologically proven carcinoma of the intrathoracic oesophagus or gastro-oesophageal junction who underwent nCRT according to CROSS between January 2002 and December 2013 followed by oesophagectomy, were identified from a prospectively collected database. The majority of the patients were treated at the Erasmus MC (n = 255, 66.9%), University Medical Centre Rotterdam, which is a tertiary referral centre for patients with oesophageal carcinoma in the Netherlands. The remaining patients (n = 126, 33.1%) were treated between 2004 and 2008 in one of the centres, which participated in the randomized CROSS trial [6]. Ethical approval was not required because of the retrospective character of the study as judged by the ethical committee of the Erasmus MC.

STAGING

Tumours were (re-)staged according to the 7th UICC-AJCC TNM staging manual [15]. All patients underwent physical examination and routine haematological and biochemical tests. An upper gastro-intestinal endoscopy with biopsies, endoscopic ultrasonography (EUS) with fine needle aspiration (FNA) when indicated, computed tomography (CT) of the neck, chest and abdomen and external ultrasonography of the neck with FNA in case of suspected lymph nodes was performed in every patient. Bronchoscopy was only indicated for tumours with suspected infiltration of the tracheobronchial tree. Positron emission tomography (PET) was used in selected cases, but was not (yet) incorporated in the pretreatment staging of all patients of the study.

SELECTION OF CLINICAL PARAMETERS

Based on the cohort size, a maximum of 11 predictive parameters was allowed to be selected for response prediction. Selection of parameters was partly based on previously published literature and partly on generally accepted eligible criteria for nCRT followed by surgery [3, 16-18].

NEOADJUVANT CHEMORADIOTHERAPY

Chemoradiotherapy prior to surgery was given within 5 weeks after completion of clinical staging and discussing the patient in the multidisciplinary team meetings. On days 1, 8, 15, 22, and 29, Carboplatin and Paclitaxel (targeted at an area under the curve of 2 mg per millilitre per minute and at a dose of 50 mg per square meter of body-surface area respectively) were administered intravenously. Concurrent with this chemotherapy a total 3D conformal radiation dose of 41.4 Gy (Gray) was given in 23 fractions of 1.8 Gy each, with 5 fractions administered per week, starting on the first day of the first chemotherapy cycle.

SURGERY

All operations were performed or strictly supervised by experienced upper GI surgeons. For tumours substantially involving the gastro-oesophageal junction or in patients with a poor performance status (WHO performance score of 2 or higher), a transhiatal resection was favoured [19,20]. A transthoracic approach with two-field lymph node dissection was generally performed for tumours of the intrathoracic oesophagus and for junctional tumours with positive lymph nodes at or above the carina. A dissection of the nodes along the celiac axis and its branches was carried out in both approaches. A gastric tube reconstruction with cervical anastomosis was the preferred technique for restoring the continuity of the digestive tract.

PATHOLOGICAL RESPONSE EVALUATION

The fresh resection specimens was sent to the Department of Pathology and immediately examined by the attending pathologist. Samples of the tumour, lymph nodes and resection margins were obtained before the specimen was fixed in formalin. The tumour was staged according to the 7th UICC-AJCC TNM staging manual [15]. A radical resection (ypR0) was defined as no tumour cells within 1 mm of the circumferential, proximal or distal resection margins. Hence, when tumour cells were detected at or within 1 mm of the resection plane it was classified as ypR1. The number of lymph nodes removed and the number of tumour positive lymph nodes removed were recorded.
The tumour regression grade (TRG), used to assess the response to nCRT was classified into four categories according to the modified Mandard score: TRG 1, no vital tumour cells in the resection specimen (pCR of the primary tumour and removed lymph nodes, ypT0N0M0); TRG 2, less than 10% vital residual tumour cells and/or any vital residual tumour cells in the lymph nodes; TRG 3, between 10 and 50% vital residual tumour cells; and TRG 4, more than 50% vital residual tumour cells [21,22]. A single pathologist evaluated all the resection specimens.

STATISTICAL ANALYSIS

The patient characteristics considered for the development of the prediction model were selected from the literature and from clinical experience [3, 16-18]. The following parameters were considered to be potentially predictive for pCR: age at diagnosis, gender, histology and location of the tumour, differentiation grade, alcohol use, smoking, percentage weight loss, Charlson Comorbidity Index (CCI) [23], cT-stage of the tumour and cN-stage of the tumour.

Differentiation grade of the tumour was dichotomized in poorly differentiated vs. moderately and well differentiated. Alcohol use was divided into none, ≤2 units per day and >2 units per day. Smoking contained three categories; none-smoking or stopped, sporadically smoking and heavily smoking (more than half a package of cigarettes per day). CCI was dichotomized into none vs. one or more comorbidities. cT-stage was coded as cT1/T2 vs. cT3/T4 and cN-stage was coded as cN0, cN1 or N2/N3. Frequencies of the individual parameters were calculated using SPSS statistical software (Version 22; IBM SPSS Inc., Armonk, NY, USA).

In the database 25% of patients had missing values for differentiation grade. For alcohol use and smoking, the percentage of missing values was 1%. For cT-stage and cN-stage, 4 (1%) and 5 (1.3%) patients respectively had missing data. Missing data were imputed 5 times using the multivariate imputation by chained equations (mice) algorithm [24].

Logistic regression was used to estimate univariable and multivariable regression coefficients and odds ratios with 95% confidence intervals for each selected parameter. The association between continuous parameters (i.e. age and percentage weight loss) and the probability of pCR was studied with restricted cubic splines with three knots [25]. The restricted cubic splines were approximated with simple transformations.

A full prediction model with all parameters included transformation was fitted in a complete dataset. Rubin's rules were used to combine the estimated regression coefficients and standard errors of the regression coefficients [26]. For variable selection backward selection was applied with a selection criterion of $p<0.20$ based on the Wald statistics of the pooled regression coefficients. The regression coefficients in the final model were multiplied by a shrinkage factor, which was obtained using bootstrapping [27]. This model was also fitted using complete case analysis as a sensitivity analysis.
The final model with shrunken regression coefficients was presented as a nomogram to facilitate clinical application. The performance of the prediction nomogram was studied in terms of discrimination. Discrimination refers to the ability of the prediction model to distinguish between patients with and without the event and was quantified using the concordance statistic (c-statistic), which is equivalent to the area under the receiver operating characteristic curve (AUC) when dichotomous outcomes are considered [28]. The developed prediction model was internally validated using bootstrapping with 1,000 samples to correct for optimism.

All analyses were done in R-3.1.2 (R: Language and Environment for Statistical Computing, 2014, Vienna, Austria). Logistic regression models were developed using the regression modelling strategies (rms) package and multiple imputation was performed using the mice package.
Survival analysis
To study the clinical relevance of a pCR on recurrence and overall survival, the Kaplan Meier method was used calculate overall survival for patients with a pCR and patients with residual disease left in their resection specimen. Moreover disease free survival was evaluated and differences in cumulative incidence of location of recurrence were also evaluated [29].

Results

A total of 381 patients, diagnosed with oesophageal cancer between December 2000 and August 2013 were included in the study. The majority of patients were male (78.7%), and the median (range) age of the study cohort was 61 (19-82) years. The majority of the tumours was an adenocarcinoma (76.9%) and was located in the distal oesophagus (69.6%). The study cohort was further characterized by an overrepresentation of cT3 tumours (76.4%) and cN1-3 tumours (64.5%) (Table 1).

Ten patients received only four cycles of chemotherapy and two patients received six cycles; all others received the planned five cycles. Two patients received 22 fractions of 1.8 Gy, one patient 24 fractions and one patient 28 fractions; all others received the planned 23 fractions. Median (range) time to surgery (time between end of nCRT and date of operation) was 48 (18-291) days. A transhiatal oesophagectomy was performed in 177 patients (46.5%), while 199 patients (52.2%) underwent a transthoracic resection. Five patients underwent a left sided transthoracic approach.

Of the 381 patients, 105 patients (27.6%) showed a pCR in the resection specimen (ypT0N0M0). No residual tumour cells were present at the site of the primary tumour (ypT0) in 128 patients, but in 16 patients lymph nodes still contained vital tumour cells. Pathological characteristics are shown in Table 2.

The univariable and multivariable associations between the considered parameters and pCR are shown in Table 3. The multivariable analysis showed that gender, tumour histology, differentiation grade, and cT-stage were the strongest predictors of pCR. A linear association between age at diagnosis and pCR was observed. The association between weight loss and pCR was subsequently modelled using a linear term between 0% and 10% weight loss and a constant term for values above 10%. The regression coefficients in the final model were multiplied with the estimated shrinkage factor of 0.75. The apparent c-statistic was 0.69 indicating a reasonable discriminative ability in predicting pCR. After correction for optimism the c-statistic dropped to 0.64 indicating the influence of some optimism. Similar estimates of predictor effects were observed when performing a complete case analysis.
The nomogram developed to predict a pathologically complete response is shown in Figure 1.

Survival analysis showed that patients with a pCR had significantly improved 5-year survival compared to patients with residual disease (70.1% versus 42.9%, $p < 0.001$). Even so disease free survival was significantly improved (60.5% versus 38.2%, $p < 0.001$). Locoregional recurrence and distant metastases occurred less frequently in patients with a pCR ($p < 0.001$ and $p = 0.004$ respectively).

Discussion

This study was set out to identify factors prior to nCRT that would predict a pathologically complete response (pCR) after neoadjuvant chemoradiotherapy. The percentage of patients with pCR was 27.6%, hence the power of this study made it possible to develop a nomogram with a maximum of eleven selected parameters. After internal validation by bootstrapping, gender, tumour histology, differentiation grade of the tumour and pretreatment cT-stage are the useful parameters pretreatment for prediction of pCR (c-statistic of 0.64 after correction for optimism).

Females, a poorly differentiated (G3 or G4) squamous cell carcinoma, and a low cT-stage (cT1 or cT2) have the highest chance of a pCR after neoadjuvant chemoradiotherapy followed by surgery. An apparent c-statistic of 0.64 indicates a reasonable discriminative ability in predicting a pathologically complete response. The findings of the present study are supported by previous publications. Squamous cell carcinomas respond better to neoadjuvant nCRT than adenocarcinomas [6, 30-32]. It has also been found that early stage tumours more often show pCR after nCRT than more advanced tumours, possibly due to the lower tumour burden [33]. A poorer differentiation grade of the primary tumour implicates a better response to nCRT. A possible explanation could be that poorly differentiated tumours are more susceptible to nCRT because of a higher turnover of cells and consequently a higher susceptibility for DNA damage and apoptosis [34]. Female gender leading to a higher probability of pCR is a striking finding, with no strong supporting evidence from the literature. However, speculations can be made. Female gender has already been related to a better survival in (oesophageal) cancer patients [35-37], suggesting that sex hormone patterns may have a role in women's superior ability to cope with (the treatment of) cancer.
The present study only included parameters known before the start of nCRT and therefore factors such as completion of the planned neoadjuvant treatment regimen or waiting time to surgery after nCRT were not taken into account [38]. Only a minority of the patients (n = 10, 2.6%) did not complete the planned treatment regimen.

This study did not evaluate the clinical response to nCRT at the end of the neoadjuvant regimen. Accurate prediction of complete response before nCRT or accurate clinical assessment of tumour response after nCRT could identify those individuals who potentially do not need surgery with its inherent morbidity and mortality. It remains difficult to assess a complete response clinically. The current restaging modalities seem to be not accurate enough, because of low sensitivity of CT and EUS for restaging [39,40], as well as a low negative predictive value up to 23 % of tumour free biopsies [41].
Combined PET-CT can also be applied for this purpose, but the optimal timing after nCRT is uncertain as well as the diagnostic accuracy in assessing pCR after nCRT [13]. PET-CT can be used, however, to detect interval metastases [42]. Radiation causes an inflammatory reaction of the oesophagus and its surrounding tissue, thus inducing FDG-uptake with a false positive interpretation. By prolonging the interval between completion of nCRT and surgery this false impact of radiation-induced inflammation can probably be diminished (Dutch Trial Registry: NTR - 4834).

Besides looking at diagnostic modalities to predict response to chemoradiotherapy in oesophageal cancer patients, it is of interest to study the role of tissue or serum biomarkers [43]. in this context. Recent studies have shown promising markers such as stem cell markers in oesophageal cancer [44] and microRNAs as predictive biomarkers for response [45]. These findings have

not yet been validated and their clinical relevance needs to be further elucidated. Prospective (randomized controlled) trials are needed to assess the accuracy and predictive value of these markers.

The impact of pCR on outcome is not evaluated in this study. From literature it is known that pathological staging, more specifically ypN-status, is the most prognostic factor for survival [4,6], but also response grades correlate with survival. Patients with a pCR have significantly improved survival, and adjacent to this also a significantly better disease free survival, where locoregional recurrences occur less often than disseminated disease [4,9,11]. Also in our studied cohort a pCR was significantly associated with these outcomes.

There are some drawbacks and limitations in this study. First, its retrospective nature with missing data; clinical TNM staging was mainly done with EUS and CT, while PET-CT scanning was optional and not standard of care. A minor subgroup of this study cohort could not be restaged retrospectively from TNM 6th edition to TNM 7th edition [15]. This lack of information may skew the study cohort. However, a valid imputation model enabled the proper generation of missing data. Secondly, although internal validation was applied by using a bootstrapping technique, external validation in an independent cohort of patients is required, before clinical application of the described nomogram can be recommended.
Despite these limitations, this study was conducted in a large cohort of patients, all treated with the same nCRT regimen (according to CROSS) prior to oesophagectomy. The designed and internally validated nomogram includes clinical parameters based on patient and tumour characteristics that can be easily obtained in clinical practice prior to the start of nCRT.

In conclusion, a nomogram predicting pathologically complete response was developed based on clinical parameters prior to the start of neoadjuvant chemoradiotherapy. This nomogram can be useful in further developing an oesophagus preserving treatment strategy for oesophageal cancer.

CONFLICT OF INTEREST STATEMENT

All authors have no conflicts of interest, nor relevant financial interests, activities, relationships, additional affiliations, and declarations.

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Chapter six

tables and figures

Table 1

Patient and tumour characteristics of 381 patients with oesophageal or junctional cancer who underwent neoadjuvant chemoradiotherapy according to CROSS followed by surgery.

Characteristic	All patients (n=381)
Age	
Median (Range)	61.12 (19 – 82)
Gender	
Male	300 (78.7%)
Alcohol consumption	
None	84 (22.0%)
≤ 2 units per day	168 (44.1%)
> 2 units per day	124 (32.5%)
Unknown	5 (1.3%)
Smoking	
None or stopped	237 (62.2%)
Sporadically/rarely	52 (13.6%)
More than ½ pack per day	90 (23.6%)
Unknown	2 (0.5%)
Charlson comorbidity index	
0	275 (72.2%)
1	106 (27.8%)
Percentage weight loss	
None	146 (38.3 %)
< 10 %	174 (45.7 %)
≥ 10 %	61 (16 %)
Tumor type	
Adenocarcinoma	292 (76.6%)
Squamous cell carcinoma	89 (23.4%)
Histopathological grading (biopsy)	
G1	10 (2.6%)
G2	157 (41.2%)
G3	118 (31.0)
Gx	96 (25.2%)
Tumor length (cm)	
Median (Range)	5 (1 – 14)
Tumor location	
Middle	56 (14.7%)
Distal	265 (69.6%)
Gastroesophageal junction	60 (15.7%)
Clinical T stage	
cT1	10 (2.6%)
cT2	67 (17.6%)
cT3	291 (76.4%)
cT4a	**8 (2.1%)**
cTx*	5 (1.3%)
Clinical N stage	
cN0	131 (34.4%)
cN1	168 (44.1%)
cN2	68 (17.8)
cN3	10 (2.6%)
cNx*	4 (1.0%)

* cT and/or cN stage could not be obtained via endoscopic ultrasonography

Table 2

Pathological assessment in the resection specimen of 381 patients with oesophageal or junctional cancer who underwent chemoradiotherapy according to CROSS followed by surgery

Characteristic	All patients (n = 381)
Tumor histology	
Adenocarcinoma	287 (75.3%)
Squamous cell carcinoma	91 (23.9%)
Other®	3 (0.8%)
Histopathological grading	
ypGx*	226 (59.3%)
ypG1	4 (1.0%)
ypG2	75 (19.7%)
ypG3	76 (20.0%)
T stage	
ypT0*	128 (33.6%)
ypT1	54 (14.2%)
ypT2	71 (18.6%)
ypT3	124 (32.5%)
ypT4a	3 (0.8%)
ypTx	1 (0.3%)
Radicality	
ypR0	354 (92.9%)
ypR1	27 (7.1%)
N stage	
ypN0	252 (66.1%)
ypN1	96 (25.2%)
ypN2	24 (6.3%)
ypN3	9 (2.4%)
M stage	
ypM0	380 (99.7%)
ypM1#	1 (0.3%)
Resected and identified lymph nodes	
Median (range)	15 (0 – 62)
Tumor regression grade Δ	
TRG 1	105 (27.6%)
TRG 2	118 (31.0%)
TRG 3	95 (24.9%)
TRG 4	63 (16.5%)

® Other is defined as not specifically adenocarcinoma nor squamous cell carcinoma; pretreatment this was not clear in the diagnostic biopsy.

* Histopathological grading could not be obtained, especially in pCR patients, who did not have residual tumour left

Peroperative identification of M1 disease (liver) and resection of this metastasis

Δ Tumor regression grade, according to a modified Mandard score [21, 22]

Table 3

Associations between the considered parameters and pathologically complete response (pCR) with their accompanying shrunken coefficient in case of being a valuable predictor for pCR

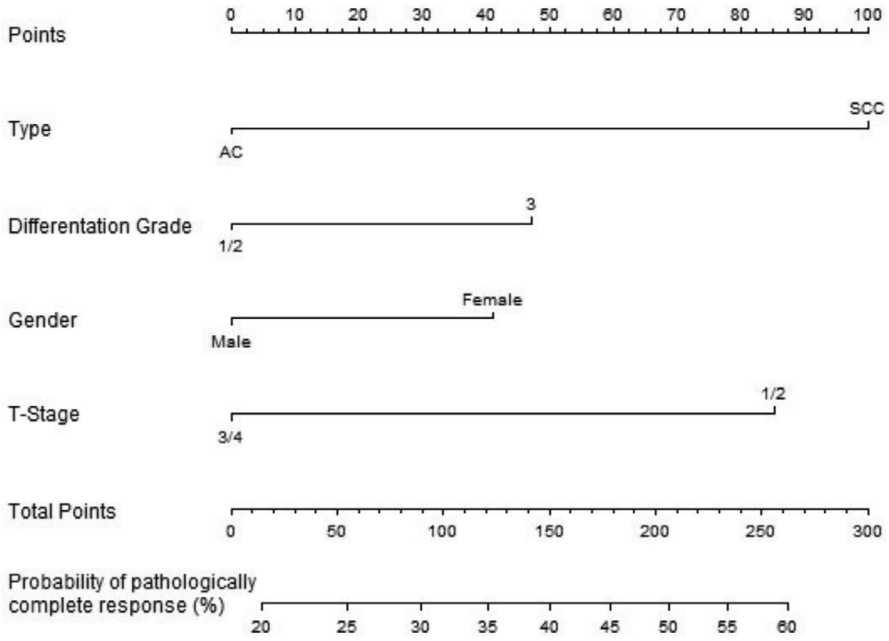
Parameter	Univariable OR* [95% CI#]	Multivariable OR* [95% CI#]	Shrunken coefficients
Age			
per decade	1.12 [0.87, 1.43]		
Gender			
Male	Ref	Ref	
Female	2.03 [1.21, 3.41]	1.53 [0.86, 2.70]	0.30
Tumor histology			
Adenocarcinoma	Ref	Ref	
Squamous cell carcinoma	3.12 [1.89, 5.16]	2.79 [1.61, 4.83]	0.72
Location			
Mid	Ref		
Distal	0.35 [0.19, 0.64]		
Junction	0.46 [0.21, 0.98]		
Histopathological grading			
Good or moderate	Ref	Ref	
Poor or undifferentiated	1.41 [0.82, 2.43]	1.68 [0.95, 2.99]	0.36
Alcohol consumption			
None	Ref		
≤ 2 units per day	1.65 [0.88, 3.07]		
> 2 units per day	1.30 [0.68, 2.52]		
Smoking			
None or stopped	Ref		
Sporadically/rare	1.14 [0.59, 2.19]		
More than ½ pack per day	0.78 [0.44, 1.37]		
Weight loss			
None	Ref		
< 10% (per percentage point)	0.96 [0.90, 1.01]		
> 10%	0.64 [0.36, 1.16]		
Charlson comorbidity index			
0	Ref		
1	0.99 [0.60, 1.63]		
cT-stage			
1 or 2	Ref	Ref	
3 or 4	0.41 [0.24, 0.70]	0.41 [0.24, 0.71]	- 0.62
cN-stage			
0	Ref		
1	0.84 [0.51, 1.40]		
2 or 3	0.92 [0.49, 1.71]		

* OR: odds ratio

CI: confidence interval

Figure 1

Nomogram for prediction of pathologically complete response (pCR)



The points listed at the top of the figure indicate the points per parameter, as listed on the left side of the figure. Add these points and match the total points on the second lowest line of the figure and read out the probability of a pCR by drawing a straight line to the lowest line what the probability of a pCR is (on a scale from 20 to 64%). Being a woman (43 points), with a poorly differentiated squamous cell carcinoma (48 points and 100 points) and low cT-stage (85 points) will give the highest chance (total points 276 = 64%) of a pCR after neoadjuvant chemoradiotherapy.

Chapter seven

Outcome of patients treated within and outside a randomized clinical trial on neoadjuvant chemoradio-therapy plus surgery for esophageal cancer: extrapolation of a randomized clinical trial (CROSS).

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Synopsis

The outcomes of patients with esophageal cancer treated within and outside a randomized clinical trial are evaluated. There is high external consistency and CROSS can be extrapolated to the daily practice involved in the treatment and care of these patients.

Abstract

BACKGROUND

Randomized clinical trials can provide high level of evidence for medical decision-making, but it is unclear if the results apply to patients treated outside such trials.
The aim of this study was to retrospectively compare outcomes of patients with esophageal cancer treated within and outside a randomized clinical trial.

METHODS

All patients who participated in a multicentre phase II-III trials on neoadjuvant chemoradiotherapy plus surgery for esophageal cancer between 2002 and 2008 (CROSS cohort) were compared with patients who underwent the same treatment outside the trial between 2008 and 2013 (post-CROSS cohort). Differences between these cohorts were analyzed with t-tests, while logistic regression models were used to evaluate adverse events. Overall and disease free survival was calculated using the Kaplan Meier method and Cox regression analyses.

RESULTS

Some 208 CROSS patients and 173 post-CROSS patients were included. Patients from the post-CROSS cohort were older, had more co morbidities and had poorer performance status. Clinical N-stage, but not cT-stage, was worse in the post-CROSS cohort. There were no statistically significant differences in adverse events (pulmonary, cardiac or anastomotic complications) or survival between the comparison cohorts.

CONCLUSION

The outcomes of patients treated with neoadjuvant chemoradiotherapy plus esophagectomy for cancer have a high external consistency and can be extrapolated to the daily practice of physicians involved in the treatment and care of esophageal cancer patients.

Discussion

Randomized clinical trials (RCTs) can provide high levels of evidence for treatment efficiency in medicine [1,2]. However, RCTs often have strict inclusion and exclusion criteria, which might limit the generalizability of an RCT to a target population. The effectiveness and safety of a treatment for a patient who does not match the eligibility criteria of the trial participants is unsure.

Participation in an RCT, especially in the treatment arm, can be beneficial to patients [3]. It is suggested that better care and closer and more frequent follow-up of the trial participants might lead to better outcomes than in non-participants. Studies that have evaluated this question report mixed results [4–7].

The ChemoRadiotherapy for Esophageal cancer followed by Surgery Study (CROSS) study is a RCT that compared outcomes after neoadjuvant chemoradiotherapy (nCRT) plus surgery with surgery alone in patients with esophageal cancer [8–10]. This study and a meta-analysis showed an improved survival of patients treated with nCRT [11]. Hence, multimodality treatment is now considered standard of care for patients with resectable esophageal cancer. Yet, little is known about the selection and outcomes of patients receiving nCRT plus surgery in the setting of standard of care compared to patients that have participated in the CROSS trial.

The aim of this study was to compare patient characteristics and outcomes of CROSS study participants with patients who underwent nCRT plus surgery outside this RCT so evaluate if outcomes remain similar.

Methods

PATIENTS

Patients with histologically proven esophageal cancer who participated in the CROSS I and II studies that ran between February 2001 and January 2004 (CROSS I) and March 2004 through December 2008 (CROSS II), respectively, were defined as the CROSS cohort [8,9]. Eight centers in The Netherlands participated in the CROSS trial. Inclusion and exclusion criteria have been reported previously [9,10]. All patients with a resectable esophageal cancer (cT1N1 or T2-T4a, No-N3, Mo tumor) and who were fit for nCRT plus surgery as judged by the responsible surgeon, medical oncologist and radiation oncologist between July 2008 and December 2013 were eligible for the post-CROSS cohort. These patients were all treated within the Erasmus MC. Already after closure of patient recruitment of the CROSS trial, before final publication of the full paper, the multidisciplinary team at the Erasmus MC considered nCRT as standard treatment based on systematic reviews. Hence patients were treated from 2008 onwards outside a study protocol. These patients were identified from an institutional database (Erasmus MC - University Medical Centre Rotterdam). Ethical approval was not applicable because of the retrospective design of the study as judged by the ethical committee of the Erasmus MC.

STAGING

All participating patients underwent history taking, physical examination, routine hematological and biochemical tests. The standard tumor staging procedures included an upper gastro-intestinal endoscopy with biopsies, endoscopic ultrasonography (EUS), and computerized tomography (CT) of the neck, chest and abdomen. EUS guided fine needle aspiration was performed only when indicated; external ultrasonography of the neck with fine needle aspiration in case of suspected metastatic lymph nodes, and bronchoscopy and positron emission tomography (PET) were used in selected patients only: Only in T3 tumors PET was of any additional value at that time, and was not yet standardized. Tumors were staged according to the tumor node metastasis (TNM) classification of the International Union Against Cancer 7th UICC-AJCC TNM staging manual [12].

NEOADJUVANT CHEMORADIOTHERAPY

nCRT was given within 5 weeks after completion of the tumor staging and after discussion at the multidisciplinary team meeting. On days 1, 8, 15, 22, and 29, carboplatin and paclitaxel (targeted at an area under the curve of 2 mg per mL per minute and at a dose of 50 mg per square meter of body-surface area respectively) were administered intravenously. Concurrently, external radiation was given up to a dose of 41.4 Gy in 23 fractions of 1.8 Gy each, with 5 fractions administered per week, starting on the first day of the first chemotherapy cycle.

SURGERY

For tumors involving the gastro-esophageal junction or in patients with a poor performance status (WHO performance score 2 or higher), a transhiatal resection was preferred [13,14]. A transthoracic approach with two-field lymph node dissection was mostly performed for tumors of the intrathoracic esophagus and for junctional tumors with positive lymph nodes at or above the carina. Dissection of the nodes along the celiac axis and its branches was performed in both approaches. A gastric tube reconstruction with cervical anastomosis was the preferred technique for restoring the continuity of the digestive tract. A minimally invasive approach was introduced since 2010 being a thoracolaparoscopic esophagectomy (McKeown) performed by a single surgeon. Lymph node dissection was similar to the open technique. An open left thoracoabdominal approach was used in some patients as part of a training program under the guidance of a teaching surgeon. The resection specimen was evaluated for residual disease. Irradicality of the tumor resection margins (R1) was defined as vital tumor cells within 1 mm of the resection margins (proximal, distal and/or circumferential). A pathologically complete response was defined as no vital tumor cells left in the resection specimen (ypToNoMo), according to a modified Mandard score system [15,16].
Complications were carefully registered for both cohorts. It is common sense to provide these data to the national esophageal cancer audit (DUCA).

FOLLOW-UP

All patients were seen in the outpatient clinic at least every three months during the first year after surgery and every 6 months during the second year. Patients were followed at least once a year in year 3, 4 and 5 after surgery. Only when a recurrence was clinically suspected, CT of the neck, chest and abdomen was performed.

STATISTICAL ANALYSIS

Differences in patient characteristics between the comparison cohorts were assessed using the Student t-test or chi-square test. When there were more than two categories within a parameter, a chi-square test for trend was used. The occurrence of adverse events was presented as frequencies, and differences in frequencies between the cohorts were calculated using t-test and presented as p-values with 5% as the level of statistical significance. Additionally, the odds of the occurrence of an adverse event in the two cohorts were calculated using univariable and multivariable logistic regression and presented as odds ratios (OR) with 95% confidence intervals (95% CI) and p-values. The multivariable regression model included adjustment for potential confounding by age (continuous variable), sex, surgical approach (transhiatal or transthoracic) and tumor stage (categorized according to the 7th TNM classification Ia, Ib, IIa. IIb, IIIa-IIIb, IIIc and IV). The probability of survival over time was estimated with the Kaplan Meier method and the log-rank test was used to assess differences between the cohorts. All patients were updated in July 2016 with regard to date of recurrence, survival and last day of follow-up. To determine variables that affected survival, a Cox regression model was used to calculate hazard ratios (HRs) with 95% CIs with adjustment for age, sex, surgical approach, complications (categorized as any or none) and tumor stage.

Results

PATIENTS

Some 208 patients (51 patients from CROSS I and 157 patients from the CROSS II study) were included in the CROSS cohort. The post-CROSS cohort consisted of 173 patients. Patients in the post-CROSS cohort were older, had more co morbidities, had poorer performance status, and clinical N-stage, but not cT-stage, was worse in the post-CROSS cohort (Table 1).
Some 14 patients who underwent neoadjuvant chemoradiotherapy did not proceed to surgery because of poor general condition or the patients' own decision. In another 19 patients, distant dissemination was present at surgical exploration or the primary tumor or lymph nodes found could not resected. These patients were excluded from the final analyses.

TREATMENT CHARACTERISTICS AND PATHOLOGY

More than 95% of patients in each cohort finished all 5 cycles of neoadjuvant chemotherapy and 23 fractions of radiotherapy and there were no statistically significant differences in completion rate between the cohorts (p=0.348 and p=0.196 respectively). The mean (standard deviation) time between end of the nCRT and surgery was 6.6 (0.1) weeks for the CROSS cohort and 7.9 (0.3) weeks for the post-CROSS cohort (p<0.001) (Table 2). Pathological tumor stage was not statistically significantly different between the cohorts (p=0.76). The percentage of patients with complete pathological response (ypT0N0M0) was 27 % (n=56) in the CROSS cohort and 28% (n=49) in the post-CROSS cohort (p=0.76).

ADVERSE EVENTS

There were no statistically significant differences in adverse events (pulmonary, cardiac or anastomotic complications), between the cohorts, except for chylothorax (supplementary data). The Odds Ratio (OR) of infectious complications was increased in the post-CROSS cohort compared to the CROSS cohort (OR 1.88, 95% CI 0.99-3.58, p=0.054), but the difference was not statistically significant (supplementary data).

SURVIVAL

Median overall survival was 44.2 months (IQR 15.2 – 64.9 months). Median overall survival in the CROSS cohort was 58.5 months (IQR 19.0 – 86.8 months) versus 35.0 months (IQR 12.9 – 51.4 months) for the post-CROSS cohort (95% CI 16.1 – 29.4).
The Hazard Ratio's (HR) of mortality were similar when comparing cohorts for overall survival, 30 and 90-day mortality, and disease free survival (Table 3). Overall 5-year survival and disease free 5-year survival were not statistically significantly different between the CROSS and post-CROSS cohort (Figure 1 and 2, log rank 0.90, overall 95% CI 39.2 – 43.8 and 0.69, overall 95% CI 39.6 – 44.5 respectively).

Discussion

This study shows similar survival rates in patients included in the CROSS trial as in those treated after this RCT after adjustment for confounders. Those who underwent nCRT plus surgery outside the CROSS trial were older, had more co-morbidities and a poorer performance status. Also more patients with T1 tumors and patients with extensive nodal disease (cN3 stage) underwent multimodality treatment, as the inclusion criteria of the CROSS-trial excluded these patients. This may indicate that the multidisciplinary team has become more liberal in selecting patients for nCRT given the confirmed effectiveness of this treatment [9,10].

The poorer performance status of the patients in the post-CROSS cohort did not translate into a decreased tolerance to nCRT. In both cohorts more than 95% of patients completed the treatment and went on to have surgery. The toxicity profile of the CROSS regimen is favorable compared to other neoadjuvant regimens including the MAGIC regimen and 5FU-Cisplatinum combination [8,17]. This could also have played a role in the decision of the multidisciplinary team to recommend nCRT, also for older and frail patients [18]. Age alone is not considered an absolute contraindication for surgery with or without neoadjuvant treatment.

Overall survival and disease free survival were not statistically significantly different between the cohorts. Also postoperative complications did not differ despite the small difference in co morbidity and performance status of the cohorts. A non-significantly higher percentage of patients underwent a transthoracic resection in the post-CROSS cohort which did not seem to translate in more pulmonary or cardiac complications, as has been reported before [13,18]. Pathological tumor stage was also not significantly different between the cohorts, which supports a high efficacy of the multimodal treatment that persists in the years after the trial was finished. Since the publication of the CROSS study, nCRT is used by more institutes in the Southwest of the Netherlands including centers that refer patients for surgery to the Erasmus MC. The fact that a pathologically complete response is obtained in a large percentage of patients in the post-CROSS cohort is indicative for the sustained efficacy of the chemoradiotherapy treatment.

The time to surgery after finishing with nCRT was somewhat longer for the post-CROSS cohort. This reflects logistic problems in the center and a less stringent planning of the operation as is usually dictated by a study protocol. A longer time to surgery may affect pathological staging but might not impact on survival as shown in the present study [19].

Enrolment in RCTs may lead to better outcomes in patients with cancer. In chronic myelocytic leukemia, the survival rate of patients within clinical trials was higher than patients outside trials [20]. An explanation could be the access to better medications, but especially the selection of healthier patients for trials. A recent paper on surgery for a benign upper gastrointestinal disease, trial participation did not affect clinical outcome [4].

One of the limitations of this present study is that the post-CROSS cohort was retrospectively evaluated and this may have introduced bias and incomplete reporting of outcome parameters including complications such as toxicity of the nCRT; increased toxicity in elderly patients with

poorer performance status and more co morbidity may have been missed. However, overall survival and mortality are unambiguous endpoints. It should be noted that the follow-up of the post-CROSS cohort was not as long as the CROSS cohort. Nevertheless, recurrence of esophageal cancer typically occurs within 2 years of surgery and most patients were followed up for more than 24 months. Changes in surgical techniques (minimally invasive techniques) and peri-operative patient management could to some extent have influenced outcomes in favor of the post-CROSS cohort. Moreover, selection bias could have occurred since patients that did not receive nCRT in the years after publication of the CROSS study could not be identified. Another weakness of this study is that both cohorts may not be completely similar due to the fact that the CROSS-cohort, acting as a control-cohort, was derived from a randomized controlled trial, wherein a variety of hospitals participated. The post-CROSS cohort was identified in retrospect from a single tertiary referral center (also participating in the original CROSS-study). Furthermore, the inclusion criteria for patients receiving neoadjuvant chemoradiotherapy were widened. Although no formal changes in the perioperative care protocol (e.g. enhanced recovery program) have taken place during the study period at the Erasmus MC, minor changes perioperative care, field-planning for radiotherapy and time between end of nCRT and surgery may have occurred with a (small) impact on the (short term) outcomes reported in this study [18,21]. It is unlikely that overall survival, the main outcome measure of this study, is affected by these factors. Finally, some tumors could not be restaged retrospectively from TNM 6th edition (CROSS I and II) to TNM 7th [12]. This may have had a small impact on the CROSS cohort regarding N-stage.

When the inclusion criteria of the CROSS trial were projected onto the patients of the post-CROSS cohort, 14 patients would not qualify for nCRT due to older age and 19 patients had a tumor length of > 8 cm. Despite this finding it is felt that, whilst there are differences in patient and tumor characteristics between the two cohorts, it is safe to apply nCRT to most patients with a resectable esophageal cancer who have been evaluated and discussed in a multidisciplinary team. In these patients the benefit in survival and harm of the multimodal treatment is likely within the same range as reported in patients participating in the CROSS trial.

CONCLUSION

The outcomes of patients treated with neoadjuvant chemoradiotherapy plus esophagectomy for esophageal cancer have a high external consistency and can possibly be extrapolated to the daily practice of physicians involved in the treatment and care of esophageal cancer patients.

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Chapter seven

tables and figures

Table 1

Patient and tumour characteristics

Patient and tumour characteristics of 381 patients, divided in a CROSS (N=208) and Post CROSS (N=173) cohort with patients with oesophageal or junctional cancer who underwent neoadjuvant chemoradiotherapy according to CROSS followed by surgery.

		CROSS	Post CROSS	
		Number (%)	Number (%)	P-value
Total		208	173	
Age	Mean (SD)	60 (0.8)	62 (0.7)	0.004
	<60	107 (51)	62 (36)	0.001
	60-65	37(18)	36 (21)	
	66-69	35 (17)	33 (19)	
	70-75	27 (13)	28 (16)	
	>75	2 (1)	14 (8)	
Sex	Male	163 (78)	137 (79)	0.8
	Female	45 (22)	36 (21)	
Comorbidity	No	162 (78)	113 (65)	0.002
	One or more	46 (22)	60 (35)	
Charlson index	0	162 (78)	110 (64)	0.007
	1	40 (19)	48 (28)	
	2	6 (3)	14 (8)	
	3			
Karnofsky performance status	60	1 (0)	0 (0)	0.000
	70	2 (1)	0 (0)	
	80	9 (4)	16 (9)	
	90	90 (44)	126 (73)	
	100	73 (35)	8 (5)	
	Missing	33 (16)	23 (13)	
Tumor length	Mean (SD)	4.5 (0.15)	5.1 (0.19)	0.008
	≤8 cm	183 (86)	154 (91)	
	> 8cm	7 (4)	16 (9)	0.02
	Missing	18 (9)	3 (2)	
Clinical T-stage	T1	1 (0)	9 (5)	0.04
	T2	30 (14)	37 (21)	
	T3	176 (85)	115 (66)	
	T4	0 (0)	8 (5)	
	Missing	1 (0)	4 (2)	
Clinical N-stage	N0	78 (37)	53 (31)	0.000
	N1	114 (55)	54 (31)	
	N2	13 (6)	55 (31)	
	N3	2 (1)	8 (4)	
	Missing	1 (0)	3 (2)	
Histology	Adenocarcinoma	160 (77)	133 (76)	0.5
	Squamous cell carcinoma	48 (23)	40 (23)	

Abbreviations:

CROSS, ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study; SD, standard deviation; SE, standard error.

Table 2

Details on treatment and pathology.
Details on treatment regimen and pathological assessment of the resection specimen of 381 patients, divided in a CROSS (N=208) and Post CROSS (N=173) cohort with patients with oesophageal or junctional cancer who underwent chemoradiotherapy according to CROSS followed by surgery.

		CROSS	Post CROSS	
		Number (%)	Number (%)	P-value
Total		208	173	
Chemotherapy	<5 cycles	4 (2)	6 (4)	0.348
	5 cycles	204 (98)	167 (96)	
Radiotherapy	<23 cycles	2 (1)	0 (0)	0.196
	23 cycles	206 (99)	173 (100)	
Weeks between end of nCRT and surgery	Mean (SD)	6.6 (0.1)	7.9 (0.3)	< 0.001
	≤6 weeks	95 (46)	48 (28)	< 0.0001
	>6 weeks	113 (54)	125 (72)	
Surgical approach	Transthoracic	92 (44)	89 (52)	0.734
	Transhiatal	116(56)	56 (33)	
	Other*	0 (0)	28 (16)	
Resection margins	R0	195 (94)	159 (92)	0.486
	R1	13 (6)	14 (8)	
ypT-stage**	T0	71 (34)	57 (33)	0.65
	T1	29 (14)	25 (14)	
	T2	41 (20)	30 (17)	
	T3	64 (31)	60 (35)	
	T4	2 (1)	1 (1)	
	Missing	1 (0)	0 (0)	
ypN-stage**	N0	144 (69)	108 (62)	0.22
	N1	45 (22)	51 (29)	
	N2	15 (7)	9 (5)	
	N3	4 (2)	5 (3)	
LN-ratio	Mean (SD)	0.065 (0.142)	0.046 (0.092)	< 0.0001
Pathological complete response***	T0N0M0	56 (27)	49(28)	0.76
Differentiation grade	Poor	26 (12)	53 (31)	< 0.0001
	Moderate	26 (12)	49 (28)	
	Good	1 (1)	3 (2)	
	Unknown including pCR	155 (75)	68 (40)	

Abbreviations: CROSS, ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study; nCRT, neoadjuvant chemoradiotherapy; SD, standard deviation; R0, tumour free resection margin; R1, tumour cells within 1mm or at the resection margin; ypT-stage, T-stage after nCRT; ypN-stage, N-stage after nCRT; pCR, pathologically complete response; LN-ratio, ratio positive resected lymphnodes divided by the number of resected lymphnodes (number between 0 and 1).

*Other approaches including minimally invasive esophagectomy and left thoracoabdominal approach
** pathological T- and N-stage after neoadjuvant chemoradiotherapy
*** pathologically complete response (ypT0N0M0)

Table 3

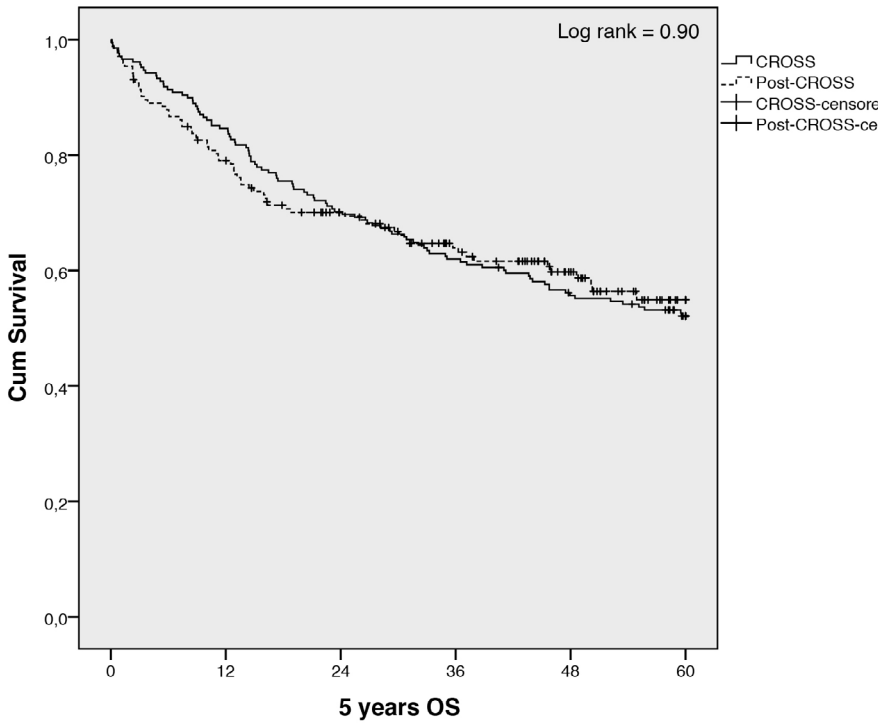
Hazard ratios for mortality comparing patients who underwent CROSS inside a trial (reference) with patients treated in the post-CROSS era.

	HR	95% CI	P-value
30-day mortality	1.37	0.40-4.68	0.62
90-day mortality	0.53	0.23-1.25	0.15
Overall survival	1.02	0.75-1.39	0.90
Disease free survival	0.93	0.67-1.31	0.69

Abbreviations:
CROSS, ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study; HR, Hazard Ratio; CI, Confidence Interval.

Figure 1

Overall survival
Overall survival of 381 patients, divided in a CROSS (N=208) and Post CROSS (N=173) cohort with patients with oesophageal or junctional cancer who underwent chemoradiotherapy according to CROSS followed by surgery (p=0.90).



	Numbers of patients at risk					
Months	0	12	24	36	48	60
CROSS (N)	208	175	145	127	112	97
Post-CROSS (N)	173	133	106	83	54	23

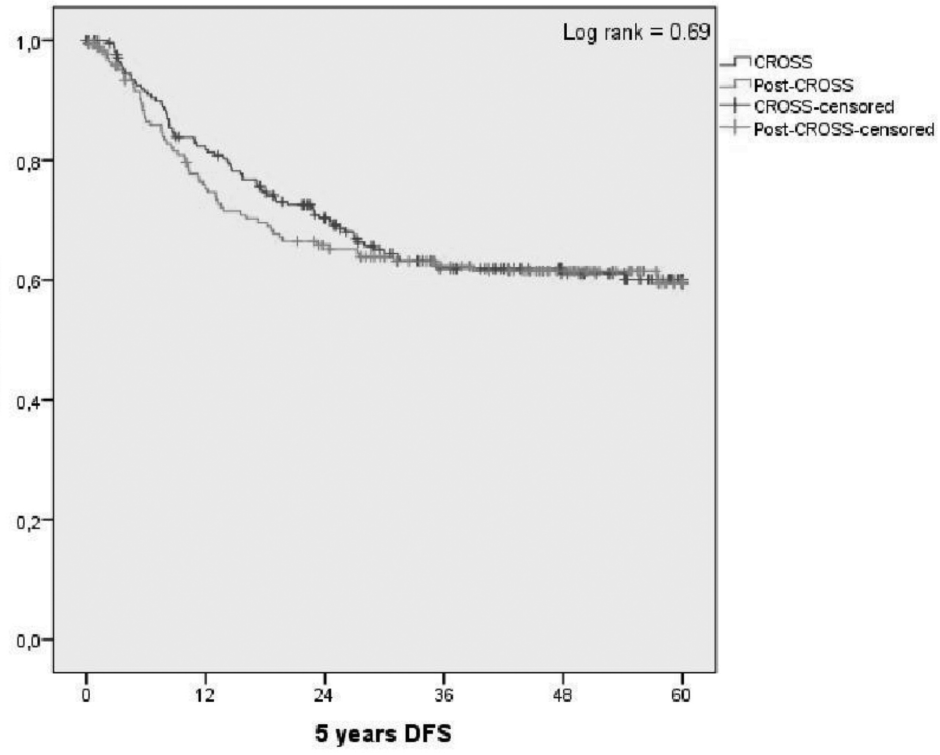
Abbreviations: CROSS, ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study; OS, Overall Survival.

Legend:
y-axis cum survival; cumulative overall survival in percentages, where 1.0 means 100% of the cohort, deceasing over time
x-axis 5 years OS; 5 years overall survival, expressed in months

Figure 2

Disease Free Survival

Disease free survival of 381 patients, divided in a CROSS (N=208) and Post CROSS (N=173) cohort with patients with oesophageal or junctional cancer who underwent chemoradiotherapy according to CROSS followed by surgery (p=0.69).



	Numbers of patients at risk					
Months	0	12	24	36	48	60
CROSS (N)	208	159	125	94	72	44
Post-CROSS (N)	173	119	99	76	55	20

Abbreviations: CROSS, ChemoRadiotherapy for Oesophageal cancer followed by Surgery Study; DFS, Disease Free Survival.

Chapter eight

Induction chemotherapy followed by surgery for advanced oesophageal cancer

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Abstract

BACKGROUND

Patients with locoregionally advanced oesophageal tumours or disputable distant metastases are referred for induction chemotherapy with the aim to downstage the tumour before an oesophagectomy is considered.

STUDY DESIGN

Patients who underwent induction chemotherapy between January 2005 and December 2012 were identified from an institutional database. Treatment plan was discussed in the multidisciplinary team. Response to chemotherapy was assessed by CT. Survival was calculated using the Kaplan Meier method. Uni- and multivariable analyses were performed to identify prognostic factors for survival.

RESULTS

In total 124 patients received induction chemotherapy mainly for locoregionally advanced disease (n=80). Surgery was withheld in 35 patients because of progressive disease (n=16) and persistent unresectability (n=19). The median overall survival of this group was 13 months (IQR: 8 - 19). The remaining 89 patients underwent surgery of which 13 still had unresectable tumour or distant metastases. Of the 76 patients that underwent an oesophagectomy, 50 patients had tumour free resection margins (66%) with an estimated 5-year survival of 37%. A positive resection margin (HR 4.148, 95% CI 2.298 – 7.488, $p < 0.0001$) was associated with a worse survival in univariable analysis, but only pathological lymph node status with increasing hazard ratio's (6.283 – 10.283, $p = 0.001$) remained significant after multivariable analysis.

CONCLUSION

Induction chemotherapy downstages the tumour and facilitates a radical oesophagectomy in patients with advanced oesophageal cancer. Pathological lymph node status is an independent prognostic factor for overall survival.

Introduction

Oesophageal cancer is the eighth most common cancer and the sixth leading cause of cancer death worldwide, with an estimated 500,000 new cases per year [1]. The overall 5-year survival rate is less than 15% due to unresectable disease or distant metastases at the time of diagnosis [2]. Chemoradiotherapy followed by surgery is considered the standard of care for non-metastasized oesophageal cancer. Five-year survival after multimodality therapy can approach 50% [3-6]. In patients with extensive and bulky locoregional disease or in cases with suspicion of metastatic disease that cannot be proven otherwise, induction chemotherapy (iCT) is indicated [7-9]. The aim of iCT is to induce tumour regression in order to make a complete resection of the primary tumour and regional lymph nodes possible [10]. The decision whether the patient is considered for induction chemotherapy is made by the multidisciplinary team and is often of empirical and pragmatic nature. This study aimed to evaluate the outcome of patients receiving induction chemotherapy for primary unresectable and incurable oesophageal cancer.

Methods

STUDY DESIGN AND PATIENT SELECTION

This is a retrospective cohort study. Patients with histologically proven carcinoma of the intrathoracic oesophagus or gastro-oesophageal junction, who underwent chemotherapy between January 2005 and December 2012, were identified from a prospective database. All patients were treated at the Erasmus MC, University Medical Centre Rotterdam, which is a tertiary referral centre for patients with oesophageal carcinoma in the Netherlands. Only patients who underwent induction chemotherapy, i.e. chemotherapy followed by response evaluation before surgery is commenced, were selected for this study. Patients who received chemotherapy with palliative intent and chemotherapy as part of definitive chemoradiotherapy or neoadjuvant chemoradiotherapy were excluded. Patients were told explicitly that the chance of surgery after the induction chemotherapy was small and only re-evaluated in patients with a favourable tumour response.

Patients that were treated with iCT reflect a population with a poor prognosis that are no candidates for primary surgical treatment or neoadjuvant chemoradiotherapy (CROSS regimen) (5) that is the treatment of choice in our unit. Indications for iCT were as follows: (1) advanced bulky locoregional disease. This group includes patients with involved large lymph nodes and/or a very bulky primary tumour with no possibility for achieving a radical resection with negative surgical margins. In these patients the extensiveness of the tumour also precludes neoadjuvant or definitive chemoradiotherapy with acceptable toxicity; (2) positive lymph nodes located outside the planned field of radiation for neoadjuvant chemoradiotherapy including the common hepatic artery in the hilum of the liver, splenic hilum or suspicious nodal disease caudal to the celiac axis; (3) clinically suspicious organ or lymph node metastases outside the surgical field (cM1 disease). The indication for iCT and other treatments was discussed at the weekly multidisciplinary team meetings (MDT), including surgeons, medical oncologists, gastroenterologists, radiologists, and radiation oncologists. Reports of the diagnostic assessments and of the multidisciplinary evaluation were carefully studied to determine the exact reason why induction chemotherapy had been given.
Ethical approval was obtained by the medical ethical committee of the Erasmus MC, University Medical Centre Rotterdam (MEC-2014-054).

STAGING

Tumours were staged according to the 7th UICC-AJCC TNM staging manual [11]. All patients underwent physical examination and routine haematological and biochemical tests. An upper gastro-intestinal endoscopy with biopsies, endoscopic ultrasonography (EUS) with fine needle aspiration (FNA) when indicated, external ultrasonography of the neck with FNA in case of suspicious malignant lymph nodes and computed tomography (CT) of the chest and abdomen was performed in every patient. Bronchoscopy was only indicated for tumours of the intrathoracic oesophagus when there was suspicion of infiltration of the tracheobronchial tree. Positron emission tomography (PET) CT was used in selected cases if there was uncertainty about N or M-stage, but was not yet incorporated in the pre-treatment staging in all patients of the study. According to the Dutch guidelines released in 2010, PET-CT is recommended for cT3 tumours.

CHEMOTHERAPY

Different regimens of iCT were applied. When the bulk of the tumour was located in the oesophagus, weekly Paclitaxel-Carboplatin or Paclitaxel-Cisplatin based regimens were preferred (six cycles) [16,17]. If the bulk of the tumour was at the gastric cardia the Epirubicin-based iCT three weekly regimen was usually given [18,19,20]. In some patients iCT was continued beyond the planned number of cycles to sustain tumour regression, or in case of partial, but still insufficient downsizing.

RESPONSE EVALUATION

After three or six cycles of iCT, response evaluation was performed by CT scan. The decision to repeat an upper gastrointestinal endoscopy or endoscopic ultrasonography (usually for fine needle aspiration of suspicious residual disease) was discussed at the weekly MDT meeting.
When there was regression of the primary tumour and/or lymph nodes patients were classified as having a partial response (PR). If no tumour was seen on the CT-scan after iCT, patients were classified as having a complete response (CR). If there was no difference in tumour and/or lymph node size after induction chemotherapy patients were classified as having stable disease (SD). In patients with progression in size of the primary tumour and/or lymph nodes or when new lesions were visible, patients were classified as having progressive disease (PD). RECIST 1.1 criteria were not used for response assessment since RECIST 1.1 correlate poorly with pathological staging [12,13]. The MDT determined, together with an experienced radiologist, how response to iCT was classified with special emphasis on the radical resectability of the tumour and lymph nodes. Surgery was proposed to the patient if there was a regression or disappearance of the lesions whereby a radical and curative resection was deemed possible by the attending surgeons. Patients without a radiological response or progressive disease were referred for further treatment with palliative intent, including radiotherapy, chemotherapy or endoscopic stent insertion.

SURGERY

All operations were performed or strictly supervised by three experienced upper GI surgeons. At the start of the operation the surgeon first determined if a macroscopically complete tumour clearance could be achieved without the presence of metastases. If this was not possible, the operation was terminated. A transthoracic approach with a two-field lymph node dissection was performed for all tumours of the intrathoracic oesophagus and for junctional tumours with positive lymph nodes at or above the carina. For tumours involving the gastro-oesophageal junction or in patients with a poor performance status (WHO performance score of 2 or higher), a transhiatal resection was favoured [14,15]. A dissection of the nodes along the celiac axis and its branches was carried out in both approaches. A gastric tube reconstruction with a cervical anastomosis was the preferred technique for restoring the continuity of the digestive tract.

PATHOLOGICAL ASSESSMENT

The tumour was staged according to the 7th UICC-AJCC TNM staging manual (11). A radical resection (ypR0) was defined as no tumour cells within 1 mm of the circumferential, proximal or distal resection margins. Hence when tumour cells were detected within 1 mm of the resection plane it was classified as ypR1. The number of lymph nodes resected and the number of tumour positive lymph nodes were recorded.
Regression of the tumour and lymph nodes, the tumour regression grade (TRG) was made based on regressive changes and the percentage vital tumour cells left in the resection specimen (16, 17). TRG was divided into four categories: TRG 1, no vital tumour cells left in the resection specimen (ypToNoM0); TRG 2, less than 10% vital residual tumour cells and/or any vital residual tumour cells in the lymph nodes; TRG 3, between 10 and 50% vital residual tumour cells; and TRG 4, more than 50% vital residual tumour cells. TRG 1 and TRG 2 are considered to be good responders, TRG 3 and TRG 4 poor responders.

FOLLOW-UP

All patients who underwent resection were followed at an interval of 3 months during the first year. In the second year, follow-up took place every six months, and yearly hereafter up to 5 years post-surgery. Only when a relapse was suspected based on clinical grounds, radiologic and/or endoscopic examinations were performed. Recurrent disease was classified as locoregional relapse, distant dissemination or both. The last date of follow up was December 1st 2013.

STATISTICAL ANALYSES

Survival and follow-up were calculated from the date of diagnosis to the date of death or date of last follow up, whichever occurred first. Follow up is reported in months with median time and interquartile ranges (IQR). The probability of survival over time was estimated with the Kaplan–Meier method and the log-rank test was used to determine statistical differences between groups. To determine which variables affects survival, a univariable Cox regression model was used. All variables with a value of $p < 0.1$ together with clinically relevant variables were included in a multivariable Cox regression model. Statistical significance was set at the 5% level (two-sided). Data are expressed as hazard ratios +/- 95% confidence intervals (CI). Statistical analysis was performed with the use of SPSS software, version 21.0 (SPSS, IBM, New York, NY, USA).

Results

PATIENTS

Between January 2005 and December 2012, 349 patients received chemotherapy and 124 patients were included in this study (Figure 1). Patient and tumour characteristics are listed in Table 1. In 20 patients (15.9%) EUS was not possible (no pass) due to tumour obstruction, which resulted in an incomplete clinical T- and N stage.
Fifty-two patients received induction chemotherapy because of advanced T- and/or N-stage (41.9%) and it was felt that a radical surgical resection was not possible. In 28 patients (22.6%), large (>2cm) unresectable celiac lymph nodes were identified, while 33 patients (26.6%) were diagnosed with positive lymph nodes outside the planned field of radiation. In nine (7.3%) patients, iCT was given for suspicious metastatic lesions and two patients (1.6%) had submucosal metastases ten centimetres or more proximal to the primary tumour.
The combination of Carboplatin and Paclitaxel was the most frequently used regimen (96 patients, 77.4%), followed by Epirubicin, Cisplatin and Capecitabine (16 patients, 12.9%). Six patients were treated with a combination of Cisplatin and Paclitaxel. Four patients switched to another regimen due to side effects. Sixty-one patients (48.4%) received their chemotherapeutic regimen without dose adjustments. Forty patients (31.7%) had postponement of one or more courses from their planned regimen due to toxicity. Adjuvant chemotherapy (chemotherapy after surgery) was not given in any of the patients.

RESPONSE EVALUATION AND PLAN OF TREATMENT

Two patients (1.6%) had a complete clinical response. A partial response was seen in 88 patients (70.9 %), nineteen patients had stable disease (15.3%) and fifteen patients had progressive disease (12.1%). Eighty-nine patients (71.8%) were referred for surgery with curative intent and 35 patients underwent further palliative treatment without surgery (Table 2). One patient refused surgery and was treated with consolidating chemoradiotherapy with curative intent (Figure 1).

SURGERY AND PATHOLOGICAL ASSESSMENT

In 76 of the 89 patients an oesophagectomy was performed, while the remaining 13 patients had surgical exploration without resection. A transhiatal approach was performed in 36 patients, while 40 patients underwent a transthoracic resection. A radical resection was achieved in 50 patients (65.8%). Tumour regression grade (TRG) was determined in the resection specimen where the majority of the patients showed a poor response of the tumour to the given induction chemotherapy (80.2%). Pathological assessment is summarized in Table 3.
Regarding the indications of treatment with induction chemotherapy, where the majority of the patients received induction chemotherapy because of locoregionally advanced disease, 28 patients had involvement of enlarged or suspect pathological coeliac lymph nodes. Ten of these patients weren't eligible for an oesophagectomy and therefore pathological stage was not available. From the eighteen patients with suspect positive coeliac lymph nodes prior to induction chemotherapy, seven had pathologically negative coeliac lymph nodes, of which two showed

significant features of tumour regression. The other eleven patients still had pathologically positive coeliac lymph nodes.
Complications occurred in 50.1% of all patients who were referred for surgery. Two patients died after surgery in the hospital, one due to severe pulmonary oedema and ARDS, the other patient due to multi organ failure preceded by an anastomotic leak.

SURVIVAL AND PROGNOSTIC FACTORS

The median survival of all 124 patients was 16 months (IQR 11 – 35). Median follow up since diagnosis was 13 months (IQR 8 -19) for patients treated with induction chemotherapy only (without oesophagectomy) and 21 months (IQR 13 - na) for patients after oesophagectomy. Patients that underwent explorative surgery only had a median survival of 11 months (IQR 9 – 16). Patients who received iCT only had a median survival of 13 months (IQR 8 -19) and two of them were still alive as of 01-12-2013 (Figure 2).
The median survival of patients after oesophagectomy (n = 76) was 21 months (IQR 13 – na) (Figure 2). The estimated five-year survival rate was 30 %. The 26 patients with tumour cells at the resection margin had a median survival of 15 months (IQR 11 -17) as compared to 39 months (IQR 19 – na) for patients (n = 50) with a microscopically tumour free resection margin ($p < 0.0001$; Figure 3).

In patients with tumour-free resection margins and no positive lymph nodes had an estimated 5 year survival rate of 72 % as compared to 21% for patients with positive lymph nodes after iCT (log rank p= 0.001). Forty-six patients (60.5%) developed metastases, either locoregional and/or distant. Disease free survival was 6 months (IQR 4.75 – 11.25). Locoregional metastases included recurrence in the gastric tube and/or locoregional lymph node metastases and occurred three patients (7%). Distant metastases were seen in 14 patients (30%). The majority of the patients however developed locoregional and distant metastases (29 patients, 63%).
Univariable analysis of factors affecting survival showed that pathological T3 stage (HR 5.753, CI 1.387 – 23.860, p = 0.02), pathological lymph node status with increasing hazard ratio's according as increasing number of positive lymph nodes and radicality of resection (HR 4.148, CI 2.298 – 7.488, $p < 0.0001$) were significantly associated with patients' prognosis. Multivariable analysis revealed pathological lymph node status (with increasing significance along increasing N-status; respectively p=0.06 for ypN1 (HR 2.983, 95% CI 0.963 – 9.237), p=0.007 for ypN2 (HR 6.283, 95% CI 1.660-23.778) and p=0.001 for ypN3 (HR 10.283, 95% CI 2.699 – 39.185)) as independent prognostic factor for survival, but also gender with a favourable prognosis for women (HR 2.411, 95% CI 1.198 – 4.851, p=0.01).

Discussion

Neoadjuvant chemoradiotherapy is considered a standard treatment in curative setting for resectable oesophageal and junctional cancer. This strategy improves survival in patients with localized oesophageal cancer. In patients with advanced T- (T4a/b) and N-stage (cN2 or more) where there is doubt about the possibility to achieve a radical resection, induction chemotherapy is given to downstage the tumour. This is followed by surgery only in patients with a favourable tumour response. It is felt that this is a good alternative to neoadjuvant chemoradiotherapy. Chemotherapy given in conjunction with radiotherapy merely acts probably as a radiosensitizer [18,19] and it is felt that a more intense systemic effect of more intense regimens of chemotherapy is important to achieve maximum tumour regression. Also in our cohort of patients pathologic lymph nodes were frequently present in the upper mediastinum and at the level of the celiac axis and even below. Trying to include these stations in the field of radiation would substantially enlarge this field and greatly impact toxicity. Some patients had signs of distant metastases that could be proven by neither histology nor cytology. Systemic chemotherapy followed by response evaluation is also a reasonable option in these patients to see if they progress (and palliation is indicated) or become amenable for surgical treatment. In the present study, nine patients (7.3%) underwent iCT for this indication. Seven of these patients showed a partial response of the primary tumour and locoregional lymph nodes, without changes of the lesions that were suspect for M1-disease and they were referred for surgery. The other two patients showed progression of the primary tumour without evidence of M1 disease and were refused for surgery. Two of the seven patients underwent explorative surgery due to unresectability (M1 or tumour overgrow) while 5 patients underwent oesophagectomy with curative intent.

The cohort described in the present study represents a relatively young population with a median age of 61 years. This reflects the stringent process of selection of these patients by the MDT. Older and frailer patients with comorbidities and similar advanced disease stages would not be amenable for this intensive treatment option and are treated with palliative intent.

It has been shown previously that the RECIST criteria correlate poor with pathological staging [12,13]. Hence it was felt that the MDT from a high volume tertiary care centre for oesophageal cancer patients is the appropriate forum for making a definite call for curative surgery or other treatment plans in these patients. Unfortunately, 15% of patients in this study scheduled for surgery were found to be unresectable/incurable. This once more underlines the limited value of CT scanning in response measurement after induction chemotherapy.

A discrepancy between clinical and pathological staging has been well described [20,21]. With the currently available staging modalities, it is rather difficult to differentiate between tumour and therapy induced reactive changes. The present study included two patients with a clinically complete response of the tumour based on CT scan. At the time of operation, one patient had peritoneal carcinomatosis and another patient showed residual tumour in the resection specimen. On the other hand, nine patients had no residual cancer (T-stage) in their resection specimen while evaluation with CT scan considered a partial response. Clearly, improved diagnostics are needed for response evaluation after induction chemotherapy [22]. Diagnostic laparoscopy and/or EUS guided FNA might be able to improve patient selection [23,24]. Other studies suggest the implementation of PET-CT, because of a higher accuracy in determining response to chemo-

therapy [22,23,25]. However, the majority of the patients that underwent a surgical exploration without oesophagectomy were found to have locally unresectable disease and PET would likely not have changed the decision-making given its low spatial resolution. Thirteen patients had stable disease after induction chemotherapy but still they were referred for surgery. In four of these patients additional diagnostic modalities were used (PET scan and endoscopy) to estimate the chances for a complete resection of the tumour. These patients were given the benefit of the doubt because of non-progressive disease and in twelve patients an actual oesophagectomy was performed. This further illustrates the complexity of tumour response measurement.
The tumour regression grade scored in the resection specimen, showed that the majority of the patients were poor responders to induction chemotherapy. In comparison to the CROSS trial 5, the present study shows lower rates of regression, possibly indicating differences in biological nature of the tumour or the additional effect of radiation in inducing tumour cell death.

This study implies that the only chance for long-term survival after iCT in patients with bulky disease is an oesophagectomy with tumour free resection margins. A median survival of 39 months can be achieved in patients with tumour free resection margins which is in the same range as published in the multimodality arm of the RTOG trial [27] and better than in the surgery alone arm of the CROSS trial [5]. Data of the published MAGIC trial [26] showed a five-year survival of 36% in the perioperative chemotherapy arm. An incomplete resection ultimately leads to fatal recurrent disease similar to patients treated with induction chemotherapy alone. The 66% ypRo resection rate in the present study is comparable to oesophageal cancer patients treated with surgery alone [5,7].
It is clinically relevant to identify factors that predict a complete tumour resection. Univariable logistic regression did not identify any factors associated with a radical resection, likely due to the small number of patients in the present study (data not shown).
Another important prognostic factor is the presence of lymph node metastases as shown in the multivariable analysis. Patients after oesophagectomy with tumour free resection margins and no positive nodes have an excellent estimated 5-year survival of 70% as compared to patients with positive lymph nodes after iCT (5 year survival 17%). This corresponds with results published in previously published articles [5,7,8,28,29].

This study has some limitations. The relatively small cohort of patients treated with iCT limits the accuracy of the multivariable analysis. This number is too small to draw firm conclusions. Yet, we confirmed once more that pathological N-stage is a strong independent prognostic factor for survival in accordance to a recently published study [30]. Pre-treatment clinical staging of the tumour was sometimes incomplete due to severe stenosis resulting in incomplete endosonography. In these patients, fine needle aspiration of suspicious lymph nodes was not always possible. This study describes the experience from a single centre which limits its external generalizability. Finally, the indications for iCT can be debated and include a heterogeneous cohort of patients. This further limits the internal as well as external validity of the study but is inevitable. Despite these limitations, this retrospective cohort study of patients treated with induction chemotherapy underlines the poor long term outcome of patients with a locally advanced oesophageal carcinoma that are not eligible for a planned surgical resection. Induction chemotherapy with the aim to downstage the tumour in order to facilitate a complete clearance of tumour is of benefit in approximately 70% patients in whom a radical oesophagectomy can be achieved with microscopically negative resection margins.

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Chapter eight

tables and figures

Table 1

Patient and tumour characteristics

Characteristic	All patients (n=124)	iCT + palliation (n=35)	iCT + surgery (n=89)
Sex			
Male	101 (81.5%)	29 (82.9%)	72 (80.9%)
Age			
Median (Range)	61 (38-77)	61 (47-76)	61 (38-77)
Tumour type			
Adenocarcinoma	89 (71.8%)	26 (74.3%)	63 (70.8%)
Squamous cell carcinoma	35 (28.2%)	9 (25.7%)	26 (29.2%)
Histopathological grading			
G1	2 (1.6%)	0	2 (2.2%)
G2	22 (17.7%)	4 (11.4%)	18 (20.2%)
G3	54 (43.5%)	18 (51.4%)	36 (40.4%)
G4	1 (0.8%)	1 (2.9%)	0
Gx	45 (36.3%)	12 (34.3%)	33 (37.1%)
Tumour length (cm)			
Median (Range)	8 (0-18)	8 (3-16)	8 (0-18)
Tumour localization			
Middle	20 (16.1%)	6 (17.1%)	14 (15.8%)
Distal	48 (38.7%)	13 (37.1%)	35 (39.3%)
Gastro-oesophageal junction	56 (45.2%)	16 (45.7%)	40 (44.9%)
Clinical T stage			
cT3	89 (71.8%)	20 (57.1%)	69 (77.5%)
cT4a	12 (9.7%)	2 (5.7%)	10 (11.2%)
cT4b	13 (10.5%)	9 (25.7%)	4 (4.5%)
cTx[a]	10 (8.1%)	4 (11.4%)	6 (6.7%)
Clinical N stage			
N0	4 (3.2%)	1 (2.9%)	3 (3.4%)
N1	25 (20.2%)	12 (34.3%)	13 (14.6%)
N2	53 (42.7%)	12 (34.3%)	41 (46.1%)
N3	28 (22.6%)	5 (14.3%)	23 (25.8%)
Nx[a]	14 (11.3%)	5 (14.3%)	9 (10.1%)
Clinical M stage			
M0	113 (91.1%)	31 (88.6%)	82 (92.1%)
Mx[a]	11 (8.9%)	4 (11.4%)	7 (7.9%)

Tumour length and location were determined with endoscopy

Clinical tumour stage (cT) was assessed with endoscopic ultrasonography (EUS) or computed tomography (CT) and was classified according to the International Union against Cancer (IUCC) tumour-node-metastasis (TNM) classification, 7th edition.

[a] T, N or M stage could not be determined (with diagnostic modalities)

Table 2

Clinical indication for surgery after induction chemotherapy in relation to tumour response

tumour response	Operation	No operation
Complete response	2	-
Partial response	74	13 (still unresectable)
Stable disease	13	6 (still unresectable)
Local progression	-	10
Distant progression	-	6
TOTAL	**89**	**35**[b]

b One patient refused surgery

Pathological TNM stage was classified according to the International Union against Cancer (IUCC) tumour-node-metastasis (TNM) classification, 7th edition. The x behind G, T, N or M implicates could not be determined, in the case of T, N or M staging. Residual tumour indicates tumour in the primary tumour and in one patient in the lymph nodes.

c Nine patients had a complete pathological response i.e. no viable tumour cells left in the resection specimen, of which one patient had tumour positive lymph nodes.

dypR1 resections: 26 patients with a tumour-positive circumferential margin and including three patients with also involvement of the proximal and/or distal margin.

e The location of the disseminated disease was a transit metastasis within the oesophagus

f Tumour regression grade where TRG 1 includes eight patients without vital tumour cells at the location of the primary tumour, not the lymph nodes. One patient did have tumour positive lymph nodes in combination with a tumour free oesophagus.

Table 3

Pathological assessment of the resection specimen in 76 patients

Characteristic	All patients (n = 76)
Tumour type	
Adenocarcinoma	56 (73.7 %)
Squamous cell carcinoma	20 (26.3 %)
Histopathological grading	
G1	2 (2.6%)
G2	23 (30.3%)
G3	40 (52.6%)
G4	0 (0%)
Gx	11 (14.5%)
Pathological T stage	
ypT0[c]	9 (11.8%)
ypTis	1 (1.3%)
ypT1a	1 (1.3%)
ypT1b	3 (3.9%)
ypT2	10 (13.2%)
ypT3	51 (67.1%)
ypT4a	1 (1.3%)
Radicality	
R0	50 (65.8%)
R1[d]	26 (34.2%)
Pathological N stage	
ypN0	21 (27.6%)
ypN1	21 (27.6%)
ypN2	16 (21.1%)
ypN3	18 (23.7%)
ypNx	0 (0%)
Pathological M stage	
ypM0	75 (98.7%)
ypM1[e]	1 (1.3%)
Resected lymph nodes	
Median	22
Range	4 - 43
Tumour regression grade[f]	
1	8 (10.5%)
2	7 (9.2%)
3	22 (28.9%)
4	39 (51.3%)

Table 4

Univariable and multivariable Cox regression analysis for survival after induction chemotherapy and oesophagectomy (n=76)

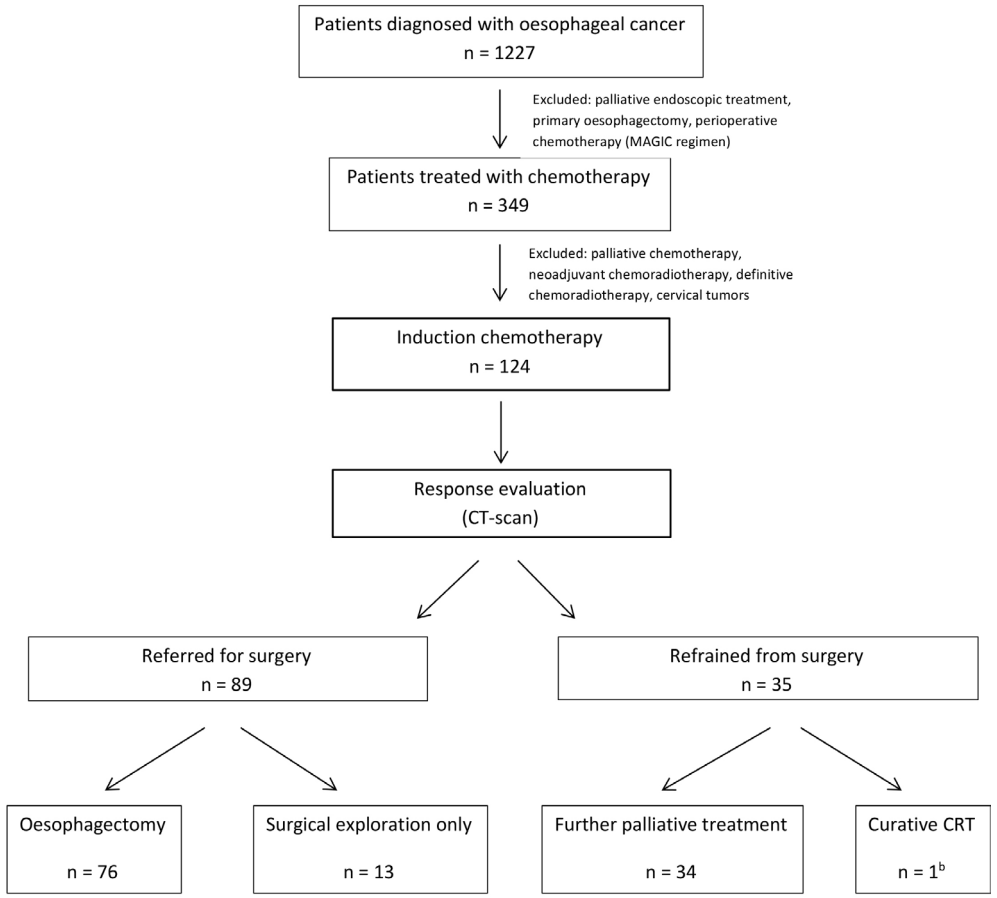
	Univariable analysis Hazard Ratio (95%CI)	P-value	Multivariable analysis Hazard Ratio (95% CI)	P-value
Gender				
Man	1		1	
Female	1.779 (0.928 – 3.410)	0.08#	2.411 (1.198 – 4.851)	0.01*
Age (yrs.)				
< 65	1			
> 65	0.954 (0.533 – 1.710)	0.88		
Tumor type				
SCC	1			
AC	0.785 (0.581 – 2.053)	0.79		
Differentiation grade				
Good	1			
Moderate	1.228 (0.153 – 9.837)	0.85		
Poor	1.850 (0.250 – 13.694)	0.55		
Unknown	1.202 (0.160 – 9.023)	0.86		
ypT-stage				
0	1		1	
1	0.868 (0.079 – 9.582)	0.91	0.547 (0.046 – 6.510)	0.63
2	2.767 (0.558 – 13.720)	0.21	1.963 (0.368 – 10.455)	0.43
3	5.753 (1.387 – 23.860)	0.02#	1.462 (0.271 – 7.876)	0.66
ypN-stage				
0	1		1	
1	3.071 (1.177 – 8.011)	0.022#	2.983 (0.963 – 9.237)	0.06
2	6.518 (2.389 – 17.786)	< 0.0001#	6.283 (1.660 – 23.778)	0.007*
3	13.771 (5.120 – 37.039)	< 0.0001#	10.283 (2.699 – 39.185)	0.001*
Radicality				
R0	1		1	
R1	4.148 (2.298 – 7.488)	< 0.0001#	1.419 (0.682 – 2.953)	0.35

Variables used in the multivariable model

* Variables that are an independent prognostic factor for survival after oesophagectomy

Figure 1

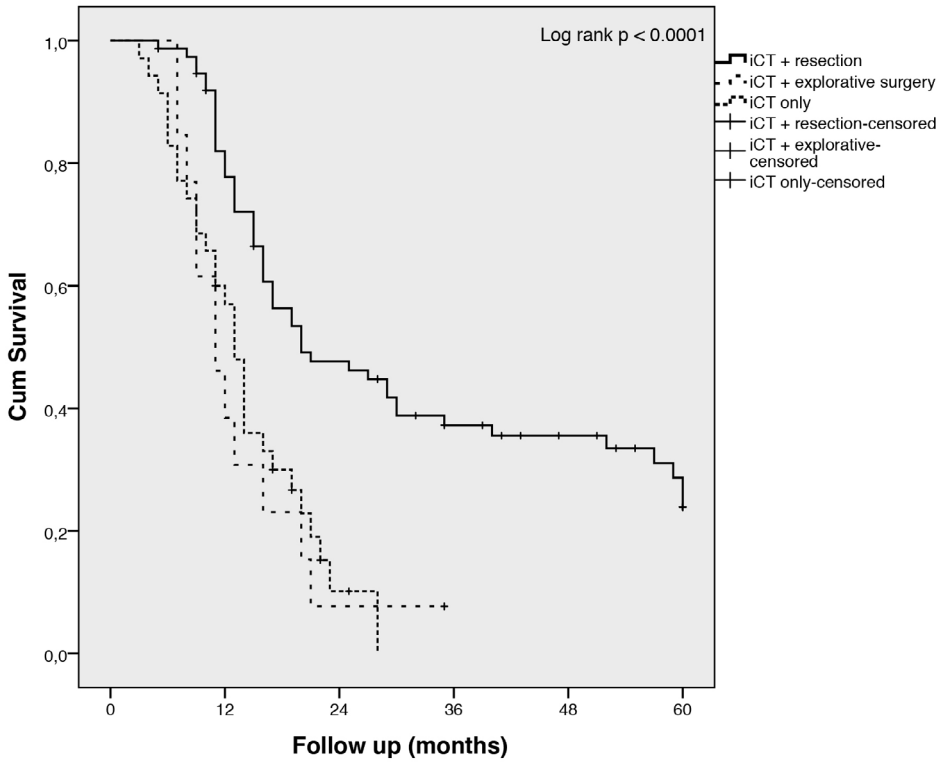
Flow diagram patient selection and follow up



b Patient refused surgery, and was referred for consolidating chemoradiotherapy instead

Figure 2

Estimated survival rates for patients after induction chemotherapy (iCT) according to treatment (n = 124, iCT alone and referred for palliation, iCT and explorative surgery, iCT and oesophagectomy).



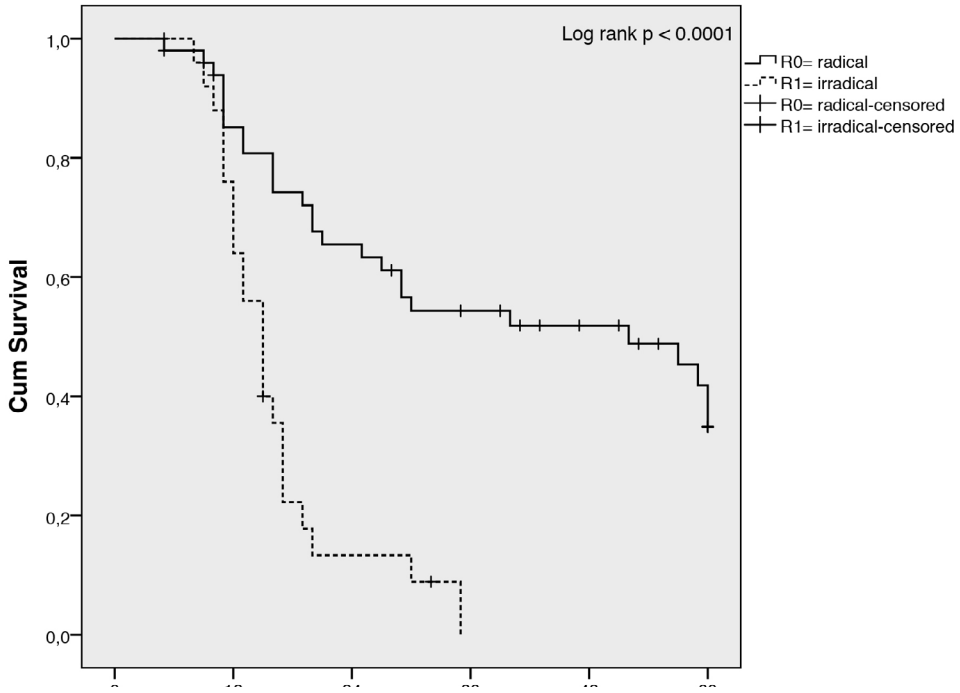
No. at risk	0	12	24	36	48	60
iCT + resection	76	62	33	25	20	13
iCT + explorative surgery	13	6	1	0	0	0
iCT only	35	20	2	0	0	0
Total	124	78	36	25	20	13

Variables used in the multivariable model

* Variables that are an independent prognostic factor for survival after oesophagectomy

Figure 3

Kaplan Meier survival curve for 76 patients who underwent oesophagectomy according to radicality of resection (ypR0 resection in 50 patients and ypR1 resection in 26 patients).



No. at risk	0	12	24	36	48	60
R0 radical resection	50	43	30	24	20	13
R1 irradical resection	26	19	3	0	0	0
Total	76	62	33	24	20	13

b Patient refused surgery, and was referred for consolidating chemoradiotherapy instead

Chapter nine

Association between paclitaxel clearance and tumor response in patients with esophageal cancer

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Abstract

BACKGROUND

Inter-individual variability in paclitaxel pharmacokinetics may play a role in the response to chemotherapy. Therefore, we studied the association between paclitaxel clearance and treatment response in patients with esophageal cancer.

PATIENTS AND METHODS

All patients who received paclitaxel (plus carboplatin) treatment for esophageal cancer between 2007 and 2013 were included. The treatment was given as neoadjuvant chemoradiotherapy (nCRT), induction chemotherapy (iCT), or palliative chemotherapy (pCT). The treatment response was assessed by the tumor regression grade (TRG) or by the RECIST1.1 criteria, respectively. The unbound paclitaxel clearance (CL) was estimated with NONMEM. The log-transformed clearance was related to response with ANOVA and independent sample t-tests.

RESULTS

A total of 166 patients were included, of whom 113 received nCRT, 23 iCT and 30 pCT. In patients receiving nCRT, paclitaxel clearance was not associated with tumor regression grade (p-value = 0.25), nor with pathologically complete response (geometric mean 561.6 L/h) and residual disease (geometric mean 566.1 L/h, p-value = 0.90). In patients who underwent iCT or pCT, also no association between paclitaxel clearance and RECIST outcome was identified (iCT: p-value = 0.08 and pCT: p-value = 0.81, respectively).

CONCLUSION

In conclusion, systemic paclitaxel exposure was not associated with response to common paclitaxel-based treatment regimens for esophageal cancer. Future studies should focus on tumor exposure in relation to systemic exposure and treatment outcome.

Introduction

The incidence of esophageal cancer is still rising in the United States and Western Europe and mortality is high [1,2]. Esophageal cancer is often diagnosed at an advanced stage. Therefore, curative treatment is only attempted in less than fifty percent of patients [3]. Based on the evidence from the Dutch randomized CROSS trial, paclitaxel can be used in combination with carboplatin and radiotherapy as an effective neoadjuvant treatment strategy [4,5]. In approximately 30% of patients, no vital tumor cells are left in the esophagectomy specimen following neoadjuvant chemoradiotherapy (nCRT) [4,6,7]. In another 30% of patients, partial regression of the tumor is observed (1–10% vital tumor cells), while in 25% of patients the resection specimen does not show changes in regression (>50% of vital tumor cells). In patients with extensive disease not amenable for surgery, induction or palliative chemotherapy (iCT or pCT, respectively) is given, where paclitaxel is also combined with carboplatin [8-11]. In this setting, the dose of paclitaxel is higher (weekly 100 mg/m2) than in the neoadjuvant setting (weekly 50 mg/m2).

Paclitaxel is a classic chemotherapeutic agent which stabilizes cellular microtubules, thereby blocking chromosomal segregation and mitosis, and eventually inducing apoptosis [12,13]. There is a suggested dose-response relationship for this agent [14,15]. Unfortunately, paclitaxel is also known for its huge inter-individual variability in pharmacokinetics, which is largely explained by (pharmaco-) genetic and environmental differences between patients [14]. As a consequence, differences in (dose-limiting) toxicities may be explained by differences in systemic exposure between patients [14,16,17]. However, if differences in outcome could also be explained by the variation in systemic paclitaxel exposure, is currently unknown.

Therefore, we hypothesized that an increased systemic paclitaxel exposure (due to low clearance) is associated with a better response to treatment for patients with esophageal cancer. Therefore, in this study, for the first time, the association between systemic exposure to paclitaxel and therapeutic effect in patients with esophageal cancer was studied.

Materials and methods

PATIENTS

All patients were treated at the Erasmus MC Cancer Institute, Rotterdam, the Netherlands, which is a tertiary referral center for patients with esophageal cancer. Patients, aged 18 years or older, treated with paclitaxel for histologically proven carcinoma of the intrathoracic esophagus or gastro-esophageal junction between November 2007 and May 2013, were identified from an institutional database (based on a prospective trial registered at www.trialregister.nl as NTR2311, study number MEC 03.264). In this study, all patients who received paclitaxel mono- or combination-therapy, were included. For pharmacokinetic purposes, a limited sampling strategy was used. All patients with esophageal cancer received either paclitaxel in a neoadjuvant chemoradiotherapy regimen, as induction treatment or in a palliative setting. For each individual patient, a treatment plan was conducted and evaluated during a weekly multidisciplinary team meeting. The ethical approval was given by the ethical committee of the Erasmus MC as an amendment to the prospective trial (NTR2311). All patients provided written informed consent for the mentioned trial.

STAGING

The tumors were (re-)staged according to the 7th UICC-AJCC TNM staging manual [25]. Every patient underwent physical examination and routine biochemical and hematological tests. In every patient, an upper gastrointestinal endoscopy with biopsies, computed tomography (CT) of the neck, chest and abdomen, and external ultrasonography of the neck with Fine-Needle Aspiration (FNA) in case of suspected lymph nodes, was performed according to the Dutch esophageal cancer guidelines. Only in T3 tumors, was Positron Emission Tomography (PET) proven to be of any additional value at that time, and was not yet standardized.

NEOADJUVANT CHEMORADIOTHERAPY

On days 1, 8, 15, 22, and 29, paclitaxel and carboplatin were administered intravenously. A paclitaxel dose of 50 mg/m2 was administered and the targeted area under the curve (AUC) was 2 mg/mL/min for carboplatin. A total 3D conformal radiation dose of 41.4 Gy was given in 23 fractions of 1.8 Gy each, with 5 fractions administered per week. Radiotherapy started on the first day of the first chemotherapy cycle [4,21].

INDUCTION OR PALLIATIVE CHEMOTHERAPY

Weekly 100 mg/m2 paclitaxel was given together with carboplatin targeting at an AUC of 4 mg/mL/min [26,27]. In some patients, induction or palliative chemotherapy was continued beyond the planned number of six cycles. This was done to either sustain tumor regression, or in case of partial response, for further downsizing tumor volume. The regimen these patients received consisted of 175 mg/m2 paclitaxel and carboplatin (targeted at an AUC of 6 mg/mL/min) and administered in three 3-weekly cycles.

SURGERY

If surgery was feasible (after neoadjuvant chemoradiotherapy or after successful induction chemotherapy), operations were performed or strictly supervised by experienced upper-GI surgeons in four hospitals, specialized in esophageal surgery. For tumors of the intrathoracic esophagus and for junctional tumors with positive lymph nodes at or above the carina, a transthoracic approach with two-field lymph node dissection was generally performed. In patients with a poor performance status (WHO performance score of 2 or higher) or for tumors substantially involving the gastro-esophageal junction, a transhiatal resection was favored [28,29].

RESPONSE EVALUATION

In patients treated with neoadjuvant chemoradiotherapy, the treatment response was based on the assessment of the resection specimen. After surgery, the resection specimens were immediately sent to the Department of Pathology and instantly examined by the attending pathologist. The samples of the tumor, lymph nodes and resection margins were obtained before the specimen was fixed in formalin. A radical resection (ypR0, where yp means pathological after neoadjuvant treatment) was defined as no tumor cells within 1 mm of the circumferential, proximal or distal resection margins [4]. Hence, when tumor cells were detected at or within 1 mm of the resection plane it was classified as ypR1. The number of lymph nodes removed and the number of tumor positive lymph nodes removed were assessed. The tumor regression grade (TRG), used to assess the response to neoadjuvant chemoradiotherapy or to induction chemotherapy, was classified into four categories according to a modified Mandard score. TRG 1 means there were no vital tumor cells in the resection specimen (pathologically complete response of the primary tumor and removed lymph nodes, ypT0N0M0); TRG 2 means there were less than 10% residual vital tumor cells and/or any residual vital tumor cells in the lymph nodes; TRG 3 means there were between 10 and 50% residual vital tumor cells; and TRG 4 means there were more than 50% residual vital tumor cells [6,30]. For this study, all samples were re-analyzed by one pathologist (K.B.).

In patients treated with induction or palliative chemotherapy, the treatment response was assessed using CT images after six weekly cycles of chemotherapy and scored according to the "response evaluation criteria in solid tumors" (RECIST) classification system [31]. A modified RECIST 1.1. score was used, where smaller lesions than required according to definitions for RECIST 1.1 were taken into account as well. All CT images were re-evaluated by a single radiologist (N.K.). If no tumor lesions were seen on the CT imaging after induction or palliative chemotherapy, the patients were classified as having a complete response (CR). When imaging showed regression of the primary tumor and/or lymph nodes or the presence of novel metastatic lesions, the patients were classified as having a partial response (PR). If there was no difference in tumor and/or lymph node size and metastatic lesions, the patients were classified as having stable disease (SD). In case of progression in size of the primary tumor and/or lymph nodes or metastatic lesions or development of new lesions, the patients were classified as having progressive disease (PD) [31].

PACLITAXEL PHARMACOKINETIC ANALYSES

The analyses for paclitaxel pharmacokinetics were performed according to previous studies [14,16,17]. In brief, from each patient, blood was taken during one of the five or six (dependent of the type of treatment) weekly chemotherapy cycles, using a formerly endorsed limited sampling strategy with 4 to 5 samples within approximately 24 hours after the start of paclitaxel infusion [14,32]. To prevent coagulation, lithium heparin was used in all samples. Subsequent to sample collection, paclitaxel concentrations were determined using a validated method [16]. Next to individual total paclitaxel plasma concentrations, a well-established population pharmacokinetic model and NONMEM software (Icon Development Solutions, Leopardstown Dublin, Dublin, Ireland) were used to determine the paclitaxel clearance (CL, L/h) in each individual patient [14].

STATISTICAL ANALYSIS

The primary outcome of this study was the association between paclitaxel clearance and response to systemic treatment in patients with esophageal cancer. The analyses of the unbound paclitaxel clearance were performed on log-transformed clearance values, since they were assumed to follow a log-normal distribution. Hence, clearance was described by means of geometric means and corresponding coefficients of variation (CV). The differences in clearance between TRG groups were tested by means of ANOVA. The post-hoc tests were only performed if the overall (omnibus) test was significant at the 5% level without correction for multiple testing. The difference between patients with a complete response (TRG1) and patients with residual disease (TRG2-4) was tested by means of the independent samples t-test. In order to interpret the difference found on the log-scale, the difference and corresponding 95% confidence interval (CI) boundaries were exponentiated to represent the geometric mean ratio and its CI on the original scale. The statistical analyses were performed with the use of SPSS software, version 22.0 (SPSS, IBM, New York, NY, USA).

Results

A total of 166 patients with esophageal cancer were included from a prospectively collected database, of whom 113 patients underwent neoadjuvant chemoradiotherapy followed by surgery. Another 23 patients received induction chemotherapy (of whom 11 proceeded to esophagectomy) and 30 patients underwent palliative treatment. Patient and tumor characteristics of all enrolled patients are listed in Table 1.

The majority of the patients was male and had an esophageal adenocarcinoma. In patients receiving neoadjuvant chemoradiotherapy, as well as induction and palliative chemotherapy: cT3 status, cN1 status, a moderately differentiated tumor, and located at the distal esophagus was seen most. Not all patients received the initially planned courses due to toxicity or the patient's condition (Table 1).

The results for individual paclitaxel clearance as the measure for paclitaxel exposure is listed per treatment and response group in Table 2. The paclitaxel clearance is expressed as the geometric mean (GM) with the coefficient of variation (CV).

Thirty-six patients who underwent neoadjuvant CRT had a pathologically complete response (32%) and 77 patients (68%) had a partial or no response, based on their esophagectomy specimen. The tumor regression grade was not significantly associated with paclitaxel clearance (p-value = 0.25, Table 2). Post-hoc tests were not performed because of the non-significant overall effect. Also, when comparing the clearance of patients with a pathologically complete response (TRG1) to the clearance of patients with residual disease (TRG 2–4) no difference was seen (geometric mean ratio = 0.99, 95% CI [0.87–1.13], p-value = 0.90, Table 3).

The radiological classification of patients—treated either by induction or by palliative chemotherapy—is also listed in Table 2. In none of the 23 patients who underwent induction chemotherapy, a progression of disease was seen. A complete response was seen in two patients, partial response in 12 patients and stable disease in nine patients. The response grade according to the modified RECIST1.1 was not statistically significantly associated with response (p-value = 0.08, Table 2). However, a possible trend was seen towards a better response in patients with an increasing paclitaxel exposure, although the number of patients with a clinical complete response was only two.

Some 30 patients treated with palliative intent were evaluated in the current analysis of whom eight patients (27%) had a progression of the disease at a moment of response evaluation after 6 cycles of chemotherapy. Also in this group, we could not identify an association between paclitaxel clearance and tumor response (p-value = 0.81, Table 2).

Discussion

To our knowledge, this is the first study that investigated the association between systemic exposure to paclitaxel and tumor response in patients with esophageal cancer. Response to paclitaxel in patients receiving neoadjuvant chemoradiotherapy (nCRT), induction chemotherapy or palliative chemotherapy was analyzed. In contrast to what was hypothesized, systemic concentrations of paclitaxel were not associated with pathological response or radiological tumor regression.

In patients receiving induction chemotherapy, a possible trend was seen towards patients with a clinical complete response having a lower paclitaxel clearance than patients with a partial response or stable disease. However, as only two patients had a clinically complete response in this subgroup, no hard conclusions can be drawn on this point.

One of the potential reasons why a relationship between pharmacokinetics and response was not seen could be that in patients receiving neoadjuvant chemoradiotherapy, the chemotherapy mainly acted as a radiosensitizer [18-20]. Thus, the effects of paclitaxel exposure on treatment outcome could be overshadowed by the combination with radiotherapy. In addition, the combination with carboplatin chemotherapy (of which no drug concentrations were measured) could have influenced the outcomes of the analyses. Furthermore, the type of tumor (adenocarcinoma versus squamous cell carcinoma) affects the response to chemoradiotherapy. Squamous cell carcinoma reacts more effectively to chemoradiotherapy, as indicated by the fact that a pathological response occurs more often in patients with squamous cell carcinomas. However, the CROSS regimen does not distinguish between the two tumor types in clinical practice [4,5,21]. In the present study, the majority of patients were diagnosed with adenocarcinoma of the esophagus, in line with the incidence in the Western world [22].

Another important reason for the lack of correlation between paclitaxel plasma pharmacokinetics and tumor response was the potential weak correlation between paclitaxel plasma exposure and paclitaxel tumor exposure. As one of its potential resistance mechanisms, a tumor may use efflux transporters (i.e. ATP-binding cassette (ABC) transporters) to limit intra-tumoral chemotherapy concentrations. Taxanes, including paclitaxel, are known substrates for these transporters [23,24]. Although we did not measure intra-tumoral drug concentrations in this study, due to its retrospective nature, we speculate that intra-tumoral paclitaxel concentrations differed substantially from plasma chemotherapy concentrations. To further explore this, we recently set up a new prospective clinical trial (the PAREO study; registered at www.trialregister.nl as NTR6356, study number MEC 16.696) in which plasma paclitaxel exposure is compared with intra-tumoral concentrations by serial tumor biopsies and simultaneous blood sampling in patients treated for esophageal cancer.

Our study has several limitations. The limited sample size of the induction and palliative treatment group could have influenced our results. However, we do think that a strong relationship between paclitaxel clearance and response still could have been detected. Nevertheless, the results of these two treatment groups should be interpreted with caution. Furthermore, not all blood samples were collected during the first treatment cycle resulting in different paclitaxel dosages, especially in the induction and palliative treatment group. However, we used clearance

as a measure for systemic exposure, which will not be strongly influenced by drug dosage. Next to this, most patients receiving palliative chemotherapy were treated with six cycles, while others received more. The response evaluation was performed after six weekly cycles (for the first time) in every patient, but the obtained blood samples for clearance were not strictly regulated to these first six weeks. This feature can be of clinical influence on the response, but the numbers were too small to characterize.

In summary, in this study, the association between systemic exposure to paclitaxel and pathological response/clinical outcome in patients with esophageal cancer was studied. The current analysis demonstrated that systemic paclitaxel exposure was not related to response to common paclitaxel-based treatment regimens for esophageal cancer. Future studies should therefore, focus on intra-tumoral exposure in relation to systemic exposure and treatment outcome.

CONCLUSION

This is the first study, which evaluated the relation of individual paclitaxel plasma pharmacokinetics and treatment response in patients with esophageal cancer treated with a regimen of chemoradiotherapy, including paclitaxel. An association between paclitaxel pharmacokinetics and response could not be demonstrated. The challenge to predict response to treatment remains highly relevant to come to true personalized medicine.

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Chapter nine

tables

Table 1

Patient and tumour characteristics

Characteristic	nCRT (n=113)	iCT (n=23)	pCT (n=30)
Sex			
Male	91 (80.5%)	16 (69.6%)	29 (96.7%)
Age			
Median years (Range)	63 (39-82)	64 (52-77)	64 (47-76)
Tumour type			
Adenocarcinoma	90 (79.6%)	13 (56.5%)	24 (80.0%)
Squamous cell carcinoma	22 (19.5%)	9 (39.1)	6 (20.0%)
Other+	1 (0.9%)	1 (4.3%)	
Histopathological grading			
G1	3 (2.5%)	0	2 (6.7%)
G2	51 (45.1%)	7 (30.4%)	5 (16.7%)
G3	32 (28.3%)	10 (43.5%)	13 (43.3%)
G4	1 (0.9%)	0	0
Gx or Missing	26 (23.0%)	6 (26.1%)	10 (33.3%)
Tumour localization			
Proximal	0	2 (8.7%)	0
Middle	18 (15.9%)	5 (21.7%)	4 (13.3%)
Distal	80 (70.8%)	10 (43.5%)	19 (63.3%)
Gastro-oesophageal junction	15 (13.3%)	6 (26.1 %)	7 (23.3%)
Clinical T stage			
cT1	4 (3.5%)*	0	0
cT2	26 (23.0%)*	0	2 (6.7%)
cT3	80 (70.8%)*	17 (73.9%)	16 (53.3%)
cT4	3 (2.7%)*	5 (21.7%)	3 (10.0%)
Missing	0	1 (4.3%)	9 (30.0%)
Clinical N stage			
N0	35 (31.0%)#	3 (13.0%)	3 (10.0%)
N1	41 (36.3%)#	5 (21.7%)	6 (20.0%)
N2	34 (30.1%)#	11 (47.8%)	10 (33.3%)
N3	3 (2.7%)#	4 (17.4%)	5 (16.7%)
Missing	0	0	6 (20.0%)
Clinical M stage			
M0	0	21 (91.3)	2 (6.7%)
M1	0	2 (8.7%)^	28 (93.3%)
TREATMENT REGIMEN			
Neoadjuvant chemoradiotherapy	113 (100%)	X	x
4 courses of Paclitaxel	3 (2.7%)	X	x
5 courses of Paclitaxel	109 (96.5%)	X	x
6 courses of Paclitaxel	1 (0.9%)	X	x

Induction or palliative chemotherapy	X	23 (100%)	30 (100%)
6 courses of Paclitaxel		8 (34.8%)	12 (40.0%)
6 + 3 courses of Paclitaxel		15 (65.2%)	17 (56.7%)
Resection	113 (100%)	11 (47.8%)	x
Other		X	1 (3.3%)£

Abbreviations:
nCRT: neoadjuvant chemoradiotherapy,
iCT: induction chemotherapy,
pCT: palliative chemotherapy

+ Other: neuroendocrine tumour
* uTstage (endosonography) in patients treated with neoadjuvant chemoradiotherapy
# uNstage (endosonography) in patients treated with neoadjuvant chemoradiotherapy
$ no location possible due to only radiological diagnostics
^ Submucosal metastasis and suspicion of lung metastasis
£ brachytherapy

Table 2

Paclitaxel clearance per treatment and response group

	Clearance (L/h) Geometric mean (CV, %)	P-value
nCRT (n=113)		0.25
TRG1 (n=36)	561.6 (34)	
TRG2 (n=28)	591.4 (20)	
TRG3 (n=37)	578.5 (29)	
TRG4 (n=12)	478.5 (56)	
iCT (n=23)		0.08
CR (n=2)	358.1 (37)	
PR (n=12)	409.9 (29)	
SD (n=9)	500.7 (8)	
PD (n=0)	X	
pCT (n=30)		0.81
CR (n=2)	488.0 (16)	
PR (n=11)	447.1 (35)	
SD (n=9)	440.5 (33)	
PD (n=8)	500.2 (23)	

Abbreviations:
nCRT = neoadjuvant chemoradiotherapy;
iCT = induction chemotherapy;
pCT = palliative chemotherapy;
CV = coefficient of variation;
TRG = tumour regression grade;
CR = complete response,
PR = partial response,
SD = stable disease,
PD = progressive disease.

Table 3

Paclitaxel clearance of patients with a pathologically complete response versus patients with residual disease after neoadjuvant chemoradiotherapy followed by surgery

	Clearance (L/h) Geometric mean (CV, %)	P-value	Geometric ratio
nCRT (n=113)		0.90	0.99
TRG 1 (n=36)	561.6 (34)		
TRG 2-4 (n=77)	566.1 (32)		

nCRT = neoadjuvant chemoradiotherapy; CV = coefficient of variation; TRG = tumour regression grade.

PART III

General

Chapter ten

General discussion and future perspectives

E.L.A. Toxopeus

General

Esophageal cancer is the eight most common cancer worldwide and the sixth most common cause of cancer-related death [1]. Incidence of esophageal adenocarcinoma is increasing rapidly in the Western world [2]. Survival is significantly improved due to addition of neoadjuvant chemoradiotherapy (nCRT), for both squamouscell carcinomas as for adenocarcinomas [3,4]. The Dutch CROSS trial reached almost 50% five-year overall survival when treating patients with locoregional non-metastasized esophageal cancer, with neoadjuvant chemoradiotherapy followed by surgery. Alas, still more than 50% of patients diagnosed with esophageal cancer cannot be treated with primary curative intent.

This thesis tried to identify clinical and biological parameters to improve outcome of patients diagnosed with esophageal cancer, either treated with curative intent, with an esophagectomy only, with induction chemotherapy with or without surgery, or with palliative chemotherapy. CMET, microRNA-126 and liver enzymes were thoroughly researched together with pharmacokinetic and clinical parameters, to identify certain leads in improving outcome when treating these patients.

Future research should focus on optimizing treatment regimens based on patient- and tumor characteristics, personalized tailor made treatment, targeted therapy and esophagus-sparing treatment, but all in order to improve survival with acceptable quality of life.

In the second chapter of this thesis, treatment of esophageal cancer is widely defined around 2012 [5]. In the following paragraphs key issues are further defined.

MOLECULAR MARKERS OR BIOMARKERS

The definition of a biomarker is a measurable indicator of some biological state or condition. It is a naturally molecule, gene, or characteristic by which a particular pathological or physiological process, disease etc. can be identified. First, it can be used for those patients who should undergo a defined treatment, so called personalized therapy. Second, to those patients in whom modifications of the multimodal therapy due to observed responses might lead to an improvement of the response and/or prognosis; individualized therapy. Third, to those patients who might not benefit from a particular toxic treatment regimen, and last for those patients who could be identified early on and thereby be spared the morbidity associated with such treatments.

In 2014 Ajani *et al.* suggested ALDH-1 as promising biomarker for predicting response to neoadjuvant chemoradiotherapy followed by surgery. ALDH-1 was identified in pretreatment tumor-biopsies [6]. Furthermore the hedgehog pathway is often upregulated in esophageal cancer and the transcriptional factor Gli-1 was predictive for a pathologically complete response in patients treated with chemoradiotherapy followed by surgery [7].

The disadvantage of using biopsies to research biomarkers, is tumor heterogeneity [8]. You have to take several biopsies from the tumor, and even surrounding (healthy) tissue to properly study tumor biology and with those potential biomarkers. In order to potentially overcome this feature, blood samples can be studied due to the fact that it is less sensitive to intra-tumoral heterogeneity.

BIOMARKERS AND LIQUID BIOPSIES

Until today, no accurate, sufficient and accessible biomarker has been found in sera to identify esophageal cancer, to monitor response to treatment, or to identify recurrence of disease, like prostate-specific antigen (PSA) or carcinoembryonic antigen (CEA) in prostate and colon cancer respectively. So called liquid biopsies are taken from patients in order to identify these specific markers. Circulating tumor cells, circulating tumor DNA, tumor exosomes, and microRNAs can be detected in this manner in sera of patients [9].

Circulating tumor DNA can be detected of patients with esophageal adenocarcinoma and is correlated to tumor load [10]. However to monitor response to treatment and to use as a marker for recurrence of disease is not been established yet. Furthermore standardization of sequencing techniques and further development of high-sensitive detection methods is highly needed. First promising steps have been taken by the author of this thesis. A prospective sera-database was set up, with patients diagnosed with esophageal cancer and treated with neoadjuvant chemoradiotherapy followed by surgery (CRTrEC study). Prior to start of neoadjuvant chemoradiotherapy, prior to each course of chemotherapy, and before and after surgery blood samples were taken from each patient. Blood samples were processed to sera and stored at -80 °C. Pilot studies were performed on these samples to test expression of for example microRNAs. This was feasible, reproducible by PCR-techniques and showed a small trend in monitoring response to neoadjuvant treatment (data not shown). Further analyses must be performed on these and additional samples to identify and validate any marker in order to use as a sustainable esophageal biomarker.

Several studies have been performed to identify a number of circulating microRNAs in initiation and progression of esophageal cancer [11].

INTRA-TUMORAL PHARMACOKINETICS

Preliminary data show that there is only a weak correlation between the plasma paclitaxel pharmacokinetics and the effectiveness of treatment [12].
The primary cause of failure of chemotherapeutic treatment in most malignancies is drug resistance of tumors. To determine the best dose for each patient, pharmacokinetics of the drug of interest in plasma can be used, but it is not directly correlated to the true efficacy of the drug [13]. Potentially, intra-tumoral drug concentrations can guide in optimizing dosage and identify those patients that are chemotherapy resistant on forehand. The Dutch PAREO trial will study the pharmacokinetics of paclitaxel by measuring concentrations in plasma and in tumor tissue. This will be done during the first and sixth week of treatment to study the pharmacokinetic resistance mechanism.

TOWARDS AN ESOPHAGUS-SPARING TREATMENT

In patients treated with neoadjuvant chemoradiotherapy followed by surgery, a pathologically complete response (no viable tumor cells found at the site of the primary tumor and locoregional lymph nodes, determined with histological examination) is the best outcome, but this must be known before proceeding to an esophagectomy.

Numerous diagnostic modalities have been evaluated to detect this pathologically complete response after neoadjuvant therapy. A systematic review and meta-analysis showed that current imaging modalities such as CT, PET-CT, and EUS seem to be insufficiently accurate to identify complete responders, with a 0.35, 0.62, 0.01 and 0.80 sensitivity respectively, whereas the pooled specificities were 0.83, 0.73, 0.99, and 0.83 [14]. Elucidating FDG-PET, it is known that this modality is not accurate enough in detecting non-responders or in predicting histological response in patients with esophageal cancer treated with nCRT [15–17]. FDG-PET is however useful to identify interval metastases, visible metastases that developed during or after nCRT, before surgery [17,18]. A recent meta-analysis showed that endoscopic biopsies, EUS, and 18F-FDG PET(-CT) as single modalities are not sufficient enough for detecting residual disease after nCRT for esophageal cancer [19]. Esophagogastroduodenoscopy with bite-on-bite biopsies and endoscopic ultrasonography (EUS) with measurement of maximum tumor thickness, together with PET-CT for interval metastases, was accurate enough for detection of residual disease after nCRT [20].
The Dutch PRIDE trial tries to develop a multimodal prediction model for histopathological response to nCRT for esophageal cancer. In the prediction model are quantitative parameters derived from MRI and 18F-FDG PET-CT scans incorporated, which will be acquired at fixed intervals before, during and after nCRT. Secondary modalities include blood samples for analysis of the presence of circulating tumor DNA (ctDNA) at 3 time-points (before, during and after nCRT), and an endoscopy with (random) bite-on-bite biopsies of the primary tumor site and other suspected lesions in the esophagus as well as an endoscopic ultrasonography (EUS) with fine needle aspiration of suspected lymph nodes after finishing nCRT.

In the Dutch phase-III multi-center, stepped-wedge cluster randomized controlled trial patients with clinically complete response are being studied. An active surveillance and surgery as needed after nCRT is compared to standard esophagectomy. This trial will lead to an esophagus-sparing approach when active surveillance is non-inferior in survival to standard esophagectomy [21].

RECOMMENDATIONS

Overall, esophageal cancer research is broadly and thoroughly studied in The Netherlands, with trials that have had or will have a major impact in treating patients with esophageal cancer (CROSS, preSANO, PRIDE, active surveillance, and PAREO).
Future studies have to focus on optimizing treatment regimens with chemotherapy and radiotherapy, towards an esophagus sparing treatment, by which molecular analysis of circulating cell-free tumor DNA derived from blood samples of all patients with esophageal cancer must be studied intensively (CRTrEC study). It is of major clinical relevance to identify those patients that will have benefit of a certain treatment, either being a good responder, to proceed with a given treatment, or being a non-responder, to change treatment and prevent patient from unnecessary risks, complications or other harms. Furthermore the biology of the tumor itself must be studied by finding intra-tumoral parameters, either by studying pharmacokinetics or at a more molecular level, to come to a more tailored made treatment. The combination of diagnostic modalities and biomarkers should provide the best prediction of responders and non-responders to treatment and overall outcome in esophageal cancer.
To achieve this goal, all must be asked to participate in studies. Blood samples and tissue that can be obtained must be stored properly and clinical relevant research questions must be set up to improve survival with acceptable quality of life in patients with esophageal cancer.

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Chapter eleven

Summary in English and Dutch

E.L.A. Toxopeus

Summary

The research described in this thesis, focusses on biological and clinical parameters to improve treatment of patients with oesophageal carcinoma. The results of this research are divided into two parts. **Part I** focusses on to potential biological parameters, examined in tissue and blood of patients with oesophageal cancer. **Part II** focusses on treatment options for patients with different stages of oesophageal carcinoma. Various patient and tumour characteristics are being evaluated in order to relate them to outcome.

Prior to this, an oversight is given about treating patient with oesophageal carcinoma. Different treatment regimens are evaluated for patients with squamous cell carcinoma, as well as for patients with adenocarcinoma, in a curative and palliative setting.

Part I

Not every tumour reacts in the same way to the given local or systemic treatment in patients with oesophageal carcinoma. This is dependent of the stage of the tumour, certain characteristics of the tumour such as differentiation, but also on patients' characteristics such as age or history. The challenge is to treat each patient with the treatment that works best against the tumour, but with the least side effects, to aim for the longest survival with a sustainable quality of life. In personalised medicine biological or molecular markers are being modified to improve effectiveness of the given treatment.

In **chapter 3** is the role of MET, the hepatocyte growth factor, evaluated in 262 patients with oesophageal adenocarcinoma. Previously published data about MET, described MET as targeted therapy to improve survival of oesophageal cancer patients. We, however, found that high expression of MET was associated with an improved overall survival compared to patients with low MET expression in their tumours. These results are obtained with a MET specific antibody, by which we think that the earlier published data, inhibiting/blocking MET, questions the rationale for using MET as a target.

The author of this thesis has devoted lots of her time to the research of microRNA's, small non coding RNA molecules that regulate gene-expression and play in that manner an important role in various cellular processes.

In **chapter 4** a diversity of functional assays are being performed to examine the role of microRNA-126 in oesophageal adenocarcinoma cell lines (OE33). MicroRNA-126 is being antagonized or overexpressed in order to perform these functional assays. MicroRNA-126 is found to play an important role in cell death in oesophageal adenocarcinoma. Furthermore, the expression of microRNA-126 was studied in 58 pre-treatment tumour biopsies, and a high expression of microRNA-126 was associated to an improved overall survival after neoadjuvant chemoradiotherapy followed by surgery.

In **chapter 5**, serum liver-derived proteins of patients with oesophageal cancer are being studied to identify a relation with survival after neoadjuvant chemoradiotherapy followed by surgery.

Also the association with response to this neoadjuvant treatment is being evaluated. None of the serum liver-derived proteins was predictive for response to neoadjuvant chemoradiotherapy. Furthermore gamma-glutamyltransferase was the only marker associated with disease free survival and an elevated level of this enzyme was also associated to disease free survival.

Part II

In chapter 6 and 7, a total of 381 patients with oesophageal carcinoma who underwent a treatment with neoadjuvant chemoradiotherapy followed by surgery are being analysed. Approximately 30% of all patients have a pathologically complete response to the neoadjuvant chemoradiotherapy. This means that there is no tumour left in the resection specimen, concluding a very effective chemoradiotherapy regimen. In **chapter 6** a nomogram is developed, predicting the chance of a pathologically complete response. Being a female, with a squamous cell carcinoma, a low T-stage of the tumour and a poorly differentiated tumour gives the highest chance of a pathologically complete response. A large part of this patient cohort participated in the CROSS trial; a randomised controlled trial where neoadjuvant chemoradiotherapy followed by surgery was compared to surgery alone. A true survival benefit was seen in patients treated with neoadjuvant chemoradiotherapy and since, this treatment regimen is considered as the golden standard in treating patients with locoregional oesophageal or junction carcinoma.

In **chapter 7** the outcome of patients participating in the CROSS trial and outside, but all treated with neoadjuvant chemoradiotherapy followed by surgery, are being compared. Patients treated outside the CROSS trial were older and frail with more comorbidities. Not all patients treated after ending of the official CROSS trial were treated within the inclusion criteria of the trial. The complications and survival were similar though, indicating that this treatment regime can be applied safely to patients outside a trial.

Less than half of the patients diagnosed with oesophageal cancer are eligible for treatment with curative intent. Five-year survival of this group of patients is approximately 50%.
In patients with locally advanced oesophageal carcinoma, or in case of suspicion of disseminated disease that cannot be proven otherwise, induction chemotherapy is proposed. In case of a good clinical response, surgery can be reconsidered. In **chapter 8** a total of 124 patients is evaluated, all receiving induction chemotherapy. Some 76 patient underwent resection after a good clinical response to the given induction chemotherapy. If patients were made eligible for surgery and the resection marges were free of tumour, a five-year survival of 37% was accomplished. Lymph node status in the resection specimen was the only independent factor associated with survival in these patients.

In **chapter 9** pharmacokinetics of paclitaxel were studied to identify a relation with response to treatment. Paclitaxel is part of the chemotherapeutic regimen received by patients with oesophageal cancer, in combination with carboplatin. In this study various groups of patients with oesophageal cancer are being evaluated; patient treated with curative intent, i.e. neoadjuvant chemoradiotherapy, patients treated with induction chemotherapy and patients treated with palliative chemotherapy. In all three groups, there was no influence of pharmacokinetics of paclitaxel on response to treatment; neither when response was measured in the resection specimen, nor when response was evaluated by CT-scans.

Nederlandse samenvatting

Het onderzoek beschreven in dit proefschrift, richt zich op biologische en klinische parameters om de behandeling van slokdarmcarcinoom te verbeteren. De resultaten worden in twee delen beschreven. **Deel I** richt zich op potentiële biologische parameters die we hebben onderzocht in weefsel en bloed van patiënten met slokdarmkanker. **Deel 2** richt zich op de behandelmogelijkheden voor patiënten met diverse ziektestadia. Er wordt gekeken of verschillende patiënt- en/of tumorkarakteristieken een relatie hebben met de prognose van de ziekte.

Voorafgaand aan deze twee delen wordt een overzicht gegeven over de behandeling van patiënten met slokdarmkanker, waarin verschillende strategieën uiteen worden gezet voor patiënten met een plaveiselcelcarcinoom en adenocarcinoom voor zowel de curatieve als palliatieve setting (hoofdstuk 2).

Deel I

Niet elke tumor reageert hetzelfde op een lokale of systemische behandeling voor slokdarmkanker. Dat heeft te maken met het stadium waarin de tumor zich bevindt, bepaalde eigenschappen van een tumor zoals de differentiatiegraad, maar ook patiëntfactoren zoals leeftijd of voorgeschiedenis spelen een grote rol. De uitdaging is om de patiënt die therapie te geven die het meest werkzaam is en met zo min mogelijk bijwerkingen om een zo lang mogelijke overleving na te streven met een goede kwaliteit van leven. In een geïndividualiseerde behandeling wordt er bekeken of bepaalde biologische parameters van de tumor gemodificeerd kunnen worden om de effectiviteit te verbeteren.

In **hoofdstuk 3** wordt de rol van MET, de hepatocyt growth factor, onderzocht in 262 patiënten met een adenocarcinoom van de slokdarm. Eerder is beschreven dat MET een aangrijpingspunt kan zijn om de overleving van patiënten met slokdarmkanker te verbeteren. Wij beschrijven echter dat hoge expressie van MET in de tumor geassocieerd is met een betere overleving hebben in vergelijking met patiënten die MET laag tot expressie brengen. Deze resultaten zijn verkregen met een specifiek antilichaam voor MET, waarbij we menen dat onze resultaten de eerder verschenen gedachten rondom MET, juist het remmen van MET expressie, in twijfel brengen.
De schrijfster van deze thesis, heeft veel tijd gestoken in onderzoek naar microRNA's; kleine niet coderende RNA-sequenties welke een belangrijke regulerende rol spelen in genexpressie. MicroRNA-126 is uitgebreid onderzocht, mede haar rol in verschillende tumoren. In hoofdstuk 4 worden diversen functionele onderzoeken verricht met een slokdarmkanker cellijn (OE33), MicroRNA-126 wordt in deze cellijn geremd of tot overexpressie gebracht waarbij er wordt gekeken naar de rol van dit microRNA. Wij vonden onder andere dat microRNA-126 een belangrijke rol speelt in celdood. Daarnaast wordt de expressie van microRNA-126 onderzocht in 58 tumorbiopten van patiënten met een adenocarcinoom van de slokdarm. Hoge expressie van microRNA-126 is geassocieerd met een betere overleving van deze patiënten na een behandeling met neoadjuvante chemoradiotherapie gevolgd door een operatie.

In **Hoofdstuk 5** onderzoeken we of serummarkers die een afgeleide zijn van de functie van de lever, geassocieerd zijn met de overleving van patiënten met slokdarmkanker. Tevens wordt gekeken of ze een rol spelen bij de respons op neoadjuvante chemoradiotherapie. Geen van de markers was voorspellend voor respons op neoadjuvante chemoradiotherapie. Gamma-glutamyltransferase was als enige marker voorspellend voor ziekte vrije overleving. Een verhoogde waarde van dit enzym gedurende de behandeling was ook geassocieerd met ziekte vrije overleving.

Deel II

In hoofdstuk 6 en 7 worden 381 patiënten geanalyseerd die een behandeling met neoadjuvante chemoradiotherapie gevolgd door een oesophaguscardiaresectie hebben ondergaan. In ongeveer 30% van de patiënten blijkt er in het resectiepreparaat geen tumor meer aanwezig te zijn, wat betekent dat de voorbehandeling met chemoradiotherapie effectief is geweest. In hoofdstuk 6 is een nomogram ontwikkeld welke de kans op een compleet pathologische respons kan voorspellen. Het bleek dat het vrouwelijk geslacht, plaveiselcelcarcinoom, een laag T stadium en slechte differentiatiegraad van de tumor de kans op een compleet pathologische respons toeneemt. Een groot deel van deze patiënten nam deel aan de zogenoemde CROSS-trial. Dit is een gerandomiseerde studie waarbij een oesophaguscardiaresectie alleen werd vergeleken met chemoradiotherapie gevolgd door een resectie. Er bleek een overlevingswinst te zijn voor patiënten die chemoradiotherapie hadden ondergaan. Sindsdien wordt deze behandelingsstrategie als de gouden standaard beschouwd voor patiënten met een locoregionaal oesophagus- of junctiecarcinoom.

In **hoofdstuk 7** zijn de uitkomsten van patiënten uit de CROSS-trial vergeleken met patiënten die na het beëindigen van de studie dezelfde behandeling hebben ondergaan.
Wat opviel was dat patiënten die na de CROSS-trial behandeld werden met hetzelfde regime, ouder waren en meer comorbiditeiten hadden. Niet alle patiënten die na de trial behandeld werden vielen binnen de inclusiecriteria van de CROSS-trial. Echter, complicaties en overleving waren gelijk in beide groepen. Dit suggereert dat dit regime (veilig) kan worden overgenomen in patiënten buiten een studie.
Minder dan de helft van de patiënten gediagnosticeerd met slokdarmkanker komt in aanmerking voor een curatief traject. De vijfjaarsoverleving is voor deze groep ca. 50%. In patiënten met uitgebreide tumorload locoregionaal of als er sprake is van een vermoeden op gemetastaseerd ziekte welke niet bewezen kan worden, wordt inductie chemotherapie voorgesteld. Bij een goede respons op deze behandeling kan een in opzet curatieve slokdarmresectie opnieuw worden overwogen. In **hoofdstuk 8** zijn in totaal 124 patiënten onderzocht welke inductiechemotherapie ontvingen. Uiteindelijk kwamen 76 patiënten in aanmerking kwamen voor een resectie na deze voorbehandeling. Als patiënten een resectie ondergingen en de resectiemarges vrij van tumor waren, dan was er een vijfjaarsoverleving van 37%. De lymfklierstatus in het resectiepreparaat was een onafhankelijke factor geassocieerd met overleving.

In **hoofdstuk 9** wordt er bekeken of de farmacokinetiek van paclitaxel iets zegt over de response van de tumor op dit chemotherapeuticum. Paclitaxel maakt deel uit van de chemotherapie die

deze patiënten ontvangen, in combinatie met carboplatin. In deze studie worden verschillende patiëntengroepen onderzocht; patiënten in een curatief traject (neoadjuvante chemoradiotherapie)en palliatief traject (alleen chemotherapie). In deze groep patiënten zagen we geen invloed van de farmacokinetiek van paclitaxel op de respons van de tumor op de voorbehandeling. Deze respons werd vastgesteld in het resectiepreparaat. In de palliatief behandelde groep patiënten zagen we ook geen relatie tussen de farmacokinetiek van paclitaxel en de respons van de tumor zoals gemeten met behulp van een CT-scan.

Chapter twelve

Acknowledgements
List of Publications
PhD portfolio
Curriculum vitae

Acknowledgements

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C. Palles, J. Jankowski et al, **E.L.A. Toxopeus** as contributing author.
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Leaving a mobilized thoracic esophagus in situ when incurable cancer is discovered intraoperatively.
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Induction chemotherapy followed by surgery for advanced oesophageal cancer.
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Exp Biology and Medicine, 2019, in press

MET protein expression in esophageal adenocarcinoma: a mislead for targeted therapy?
E.L.A. Toxopeus, J. Shapiro, M.J. de Herdt, F.A.L.M. Eskens, B. van der Steen, P.E. de Ruiter, F.J.W. ten Kate, L.J.W. van der Laan, L.H.J. Looijenga, B.P.L. Wijnhoven.
Submitted

The prognostic value of preoperative serum gamma-glutamyltransferase in patients treated for oesophageal adenocarcinoma.
K.A. Zwiers, **E.L.A. Toxopeus**, D. Nieboer, V.M.C.W. Spaander, S.M. Lagarde, A. van der Gaast, J.J.B. van Lanschot, B.P.L. Wijnhoven, L.J.W. van der Laan.
Submitted

Phd Portfolio

Name PhD student:	Eelke Toxopeus
Erasmus MC department:	Surgery
PhD period:	January 2012 – March 2015
Title thesis:	Biological and clinical parameters to improve outcome in oesophageal cancer
Promotores:	Prof. L.J.W. van der Laan and Prof. dr. J.J.B. van Lanschot
Copromotor:	Dr. B.P.L. Wijnhoven
Date PhD defense:	2 October 2019

PHD TRAINING

Courses (total 6.0 ECTS)

2014 (0.3 ECTS)	Photoshop and Illustrator CS 6 Workshop
2014 (0.6 ECTS)	Integriteitscursus, Erasmus MC, Rotterdam
2013 (1.0 ECTS)	United European Gastroenterology (UEG) Basic Science Course: "Use of human tissue in gastroenterology research", London
2013 (1.5 ECTS)	Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK) cursus, Erasmus MC, Rotterdam
2012 (0.7 ECTS)	Biostatistical Methods, Netherlands Institute for Health Sciences (NIHES), Erasmus MC, Rotterdam
2012 (0.6 ECTS)	Stralingshygiëne cursus 5A, Erasmus MC, Rotterdam
2012 (1.0 ECTS)	English Writing Course, Erasmus MC, Rotterdam
2011 (0.3 ECTS)	Minicursus voor Methodologie van Patiëntgebonden onderzoek enVoorbereiding van Subsidieaanvragen, Erasmus MC, Rotterdam

Oral presentations (0.5 ECTS each)

2014	Nomogram voor het voorspellen van een pathologisch complete respons na neoadjuvante chemoradiotherapie in het slokdarmcarcinoom. *Wetenschapsdag Heelkunde Erasmus MC, Rotterdam*
2014	- Expressie van microRNA-126 is gecorreleerd aan overleving na chirurgie bij patiënten met een adenocarcinoom van de slokdarm. - Nomogram voor het voorspellen van een pathologisch complete respons na neoadjuvante chemoradiotherapie in het slokdarmcarcinoom. *Nederlandse Vereniging voor Heelkunde (NVvH), Najaarsvergadering, Utrecht*

2014	- Induction chemotherapy followed by surgery for advanced oesophageal cancer. - c-MET protein expression in esophageal adenocarcinoma and the relation with survival; a (mis)lead for targeted therapy? *International society for diseases of the esophagus (ISDE), Vancouver, Canada*
2013	Induction chemotherapy followed by surgery for primarily incurable oesophageal carcinoma in the Netherlands. *Nederlandse Vereniging voor Gastroenterologie (NVGE), Najaarsvergadering, Veldhoven*
2013	MicroRNAs as response prediction markers to chemoradiotherapy in oesophageal cancer. *World Organization for Specialized Studies on the diseases of the Esophagus (OESO), Parijs, Frankrijk*
2013	Genome-wide screening of microRNAs as predictors for response to neoadjuvant chemoradiotherapy in oesophageal adenocarcinoma. *NVGE, Voorjaarsvergadering, Veldhoven*
2012	MicroRNas as biomarkers for the response to neoadjuvant chemo radiotherapy in oesophageal cancer. *Stafdag Heelkunde Erasmus MC, Rotterdam, Nel Kreeft prijs gewonnen*
2012	MicroRNAs en respons op neoadjuvante chemoradiotherapie in slokdarmkanker. *Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Amsterdam*
2012	MicroRNAs en respons op neoadjuvante chemoradiotherapie in slokdarmkanker. *NVvH, Voorjaarsvergadering, Veldhoven*

Poster presentations (0.3 ECTS each)

2014	MicroRNA-126 expression in pre-treatment biopsies is prognostic for survival after neoadjuvant chemoradiotherapy and surgery in patients with esophageal adenocarcinoma. *International society for diseases of the esophagus (ISDE), Vancouver, Canada*

2013	Induction chemotherapy followed by surgery for primarily incurable oesophageal carcinoma. *European Society for Diseases of the Esophagus (ESDE), Rotterdam*
2013	Induction chemotherapy followed by surgery for primarily incurable oesophageal carcinoma. *European Cancer Organisation (ECCO), Amsterdam*
2013	Induction chemotherapy followed by surgery for primarily incurable oesophageal carcinoma. *World Organization for Specialized Studies on the diseases of the Esophagus (OESO), Parijs, Frankrijk*
2012	The search for microRNAs as biomarkers for the response on chemoradiotherapy in oesophageal adenocarcinoma. *International society for diseases of the esophagus (ISDE), Venetië, Italië*
2012	The search for microRNAs as biomarkers for the response on chemoradiotherapy in oesophageal adenocarcinoma. *NVGE, Voorjaarsvergadering, Veldhoven*
2012	The search for microRNAs as biomarkers for the response on chemoradiotherapy in oesophageal adenocarcinoma. *MolMed Erasmus MC, Rotterdam*

Teaching

2013-2015 (3.0 ECTS)	Begeleiden van drie 3e jaars Hoger Laboratorium Onderwijs (HLO) studenten, per student een half jaar
2013 (3.0 ECTS)	Begeleiden 6e jaars geneeskunde student tijdens keuze-onderzoek
2012 (1.5 ECTS)	Tutor 1e jaars geneeskunde studenten

International conferences

2014 (1.0 ECTS)	International Society for Disease of the Esophagus, Vancouver, Canada
2013 (0.3 ECTS)	European Cancer Organization, Amsterdam, the Netherlands
2013 (1.0 ECTS)	European Society for Disease of the Esophagus, Rotterdam, the Netherlands
2012 (1.0 ECTS)	International Society for Disease of the Esophagus, Venice, Italy

Scientific meetings

2012-2015 (1.0 ECTS)	Voorjaarsvergadering NVGE
2012-2015 (1.0 ECTS)	NVvH voorjaars- en najaarsvergadering

Curriculum vitae

Eelke Toxopeus werd op 24 maart 1985 geboren in Eindhoven. Op haar achtste verhuisde zij naar Den Haag, alwaar zij in 2003 haar gymnasium diploma haalde aan het Gymnasium Haganum. Initieel wilde zij veearts worden, maar werd uitgeloot en startte met biomedische wetenschappen aan de Universiteit Utrecht. In 2005 startte zij dan toch met de opleiding Geneeskunde, welke in 2011 cum laude werd afgerond. Na enkele maanden als arts assistent te hebben gewerkt in het Ikazia ziekenhuis, onder supervisie van Dr. P.T. den Hoed, startte zij in 2012 met haar translationele en klinische promotietraject wat tot dit proefschrift heeft geleid. In het jaar 2014 is zij enkele maanden naar Dublin geweest om onder supervisie van Dr. J. O'Sullivan en Prof. J. Reynolds onderzoek te doen naar microRNAs en hun rol in metabolisme in slokdarmkanker. In 2015 startte zij weer met klinisch werk, opnieuw in het Ikazia ziekenhuis, waarna in januari 2016 begonnen werd met de opleiding tot chirurg in het Reinier de Graaf Gasthuis te Delft, onder supervisie van Dr. M. van de Elst en later Dr. M. de Vries. In 2018 startte zij haar academische opleidingsjaar in het Erasmus MC, onder supervisie van Dr. B. Wijnhoven, alwaar ook dit proefschrift afgerond werd. In 2020 zal zij aanvangen met de differentiatie kinderchirurgie onder supervisie van Dr. J. Vlot. Zij woont met partner Oliver en dochtertje Emily in Rotterdam.