Toward treatment optimization for patients with pancreatic and esophagogastric cancer

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TOWARD TREATMENT OPTIMIZATION FOR PATIENTS WITH PANCREATIC AND ESOPHAGOGASTRIC CANCER

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"Om de mensen met kanker weer wat hoop te geven".

Contents

1

Introduction and outline of this thesis

The overall aim of this thesis is to improve the treatment methods for pancreatic and esophagogastric cancer.

Part One: Pancreatic cancer

The incidence of pancreatic cancer in the Netherlands has doubled in the last 30 years to approximately 2800 new yearly cases and patients face the lowest survival rate of all solid tumors in the Netherlands according to the Netherlands Cancer Registry (NCR), Netherlands Comprehensive Cancer Organization (https://www.cijfersoverkanker.nl). Most patients present with either locally advanced (unresectable) or metastatic disease (1) and only 15 to 20% of patients are eligible for up-front surgery (2). Known risk factors for pancreatic cancer are a genetic predisposition and environmental risk factors including tobacco use, diet, alcohol consumption, and high caloric intake (3, 4). Staging and management of pancreatic cancer is multidisciplinary. Resection alone typically results in a 5-year overall survival (OS) rate of approximately 10%. However, the prognosis for patients with resected pancreatic ductal adenocarcinoma (PDAC) was notably enhanced with the addition of adjuvant chemotherapy with gemcitabine (5). Adjuvant chemotherapy with 5-fluorouracil (5-FU), folinic acid, irinotecan and oxaliplatin (modified FOLFIRINOX) vs gemcitabine further improved median OS (53.5 vs 35.5 months) after 5 years follow up (6). The optimal neoadjuvant treatment schedule with either chemotherapy alone or chemotherapy in combination with radiotherapy (CRT) for patients with resectable and borderline resectable tumours is under investigation and preferably given in the context of a clinical trial. Neoadjuvant gemcitabine-based CRT achieved a 15% improvement of 5 year OS for patients with resectable or borderline resectable tumors in the PREOPANC trial (7). The PREOPANC-2 trial compared total neoadjuvant FOLFIRINOX versus neoadjuvant gemcitabine-based CRT and adjuvant gemcitabine in patients with the same tumor characteristics as in the PREOPANC-1 trial and interestingly neoadjuvant FOLFIRINOX did not improve OS or resection rates compared to gemcitabine based CRT (OS 21.9 vs. 21.3 months; HR 0.87; 95% CI 0.68-1.12, p=0.28; resection rates 77% vs. 75%, p=0.69) (8).

Pancreatic cancer without distant metastasis is unresectable when there is over a 270° encasement of the superior mesenteric or portal vein, or more than 90° tumor

contact with the superior mesenteric artery, celiac trunk, or common hepatic artery (9) (https://dpcg.nl). This stage is referred to as locally advanced pancreatic cancer (LAPC). Patients diagnosed with either LAPC (unresectable, without distant metastasis) or metastatic pancreatic cancer are typically not considered candidates for curative treatment. Instead, palliative chemotherapy is recommended to enhance survival and alleviate cancer-related symptoms. However, for patients with LAPC, the option of resection may be reconsidered following chemotherapy based on their response to treatment. In a non-randomized study around 12% of patients with LAPC underwent surgical resection after treatment with FOLFIRINOX (10). For patients with LAPC or metastatic disease, FOLFIRINOX and nab-paclitaxel plus gemcitabine are currently widely used regimes. In the Netherlands the preferred first-line treatment for fit patients is FOLFIRINOX, as stated in the recently updated version of the Dutch pancreatic cancer guideline (https://richtlijnendatabase.nl/richtlijn/pancreascarcinoom). FOLRIFINOX resulted in an improvement in response rate (RR), progression free survival (PFS) and OS compared to gemcitabine monotherapy (median OS of 11.1 vs. 6.8 months) for patients with metastatic PDAC (11). Gemcitabine in combination with nab-paclitaxel showed an improved RR, OS and PFS compared to gemcitabine monotherapy (median OS of 8.5 vs. 6.7 months) (12). For patients with a known germline BRCA1/2 mutation olaparib maintenance therapy can be considered although no significant OS benefit was observed in a recent update of the POLO trial (13). The optimal second-line systemic therapy approach for patients in a good clinical condition after first-line FOLFIRINOX failure is not known. An OS of 11.5 and 12.4 months from diagnosis was reported for gemcitabine based second-line therapy after FOLFIRINOX resistance in a retrospective cohort study (14). Liposomal irinotecan and 5-FU/leucovorin is approved for use in patients with metastatic PDAC previously treated with gemcitabine-based therapy. This approval is based on the observed improvement in median OS compared to treatment with 5-FU/ leucovorin alone in the final OS analysis of the global phase 3 NAPOLI-1 trial (OS 6.2 vs 4.2 months; hazard ratio [HR] 0.75) (15). In a previous Dutch NCR cohort the median OS was 11.2 months for patients who were treated with all types of systemic second-line therapy (16). Best supportive care is considered for patients in a moderate condition.

Part one of this thesis describes two studies exploring therapeutic interventions in patients with pancreatic cancer. In **chapter 2** we focus on patients with LAPC.

Patients with LAPC were historically treated with gemcitabine monotherapy (17). CRT was extensively studied in patients with LAPC with conflicting results and did not consistently show survival benefit compared to chemotherapy alone (18, 19). At the time of the study outlined in chapter two, CRT was considered a standard treatment approach for LAPC. This was because FOLFIRINOX had not yet been widely adopted in clinical practice. In an effort to increase the sensitivity of CRT, we conducted a phase I trial that incorporated treatment with an epithelial growth factor receptor (EGFR) inhibitor based on the following considerations. In human pancreatic cancer cells overexpression of EGFR is correlated with rapidly progressive disease (20, 21) and inhibition of the EGFR pathway using erlotinib was associated with a modest survival benefit in metastatic PDAC when combined with gemcitabine (22). PDAC is known for its high (>90%) oncogenic KRAS mutation phenotype (23, 24), and while in colorectal cancer the clinical efficacy of antibodies against EGFR is restricted to patients without an activating KRAS or BRAF V600E mutation (25, 26), several preclinical studies showed that EGFR pathway inhibition can improve the radiosensitivity of pancreatic tumour cells independent of KRAS mutation status. Indeed, inhibiting the EGFR-PI3K-AKT pathway as well as the HRAS signaling pathway are potential mechanisms through which blocking of EGFR signaling can reduce cell repair capacity following radiotherapy regardless of downstream activating KRAS mutations (27, 28). These data together with preclinical studies demonstrating that the radiosensitizing activity of gemcitabine may be further enhanced by EGFR pathway inhibition, provided the rationale to explore the addition of EGFR inhibition to CRT with gemcitabine (29). Panitumumab, an EGFR specific monoclonal antibody, approved for the treatment of metastatic colorectal cancer, is generally well tolerated although skin toxicity, hypomagnesemia, and diarrhea are commonly observed (30). In order to improve the prognosis of patients with LAPC and based on the above described preclinical data we studied the addition of panitumumab to gemcitabine based CRT. The results of this dose finding and feasibility phase I trial are described in **chapter 2**.

FOLFIRINOX is the preferred multi-agent cytotoxic regimen for the initial treatment of patients with metastatic PDAC and LAPC who are in good overall condition. In the landmark study of Conroy et al. in 2011 the median number of treatment cycles of first-line FOLFIRINOX administered was 10 (range, 1 to 47)(11). In clinical practice the maximal number of cycles in patients with stable disease or response is commonly limited to 12 cycles to reduce toxicity. In daily practice, reintroduction

of FOLFIRINOX is frequently considered when disease progression occurs after a therapy free period of 4-6 months since the last FOLFIRINOX administration. Data supportive of the efficacy of this approach are however limited. In **chapter 3**, we investigated the reintroduction of FOLFIRINOX in patients who had previously benefited from first-line FOLFIRINOX treatment for both metastatic and non-metastatic PDAC. This real-world cohort study utilized data from the NCR with the aim to find the most suitable patient category for this reintroduction approach.

Part two: Esophageal and gastric cancer

Around 4000 patients were diagnosed in 2022 with esophageal and gastric cancer in the Netherlands. While the incidence of adenocarcinoma of the esophagus is increasing, the incidence of squamous cell carcinoma of the esophagus is decreasing. The incidence of gastric carcinoma also decreased from around 2000 new patients yearly in 1998 to 1100 patients in 2022. Around one third of patients with esophageal cancer (EC) and nearly half of patients with gastric cancer (GC) are diagnosed in an advanced stage without curative options according to data from the NCR, IKNL (https://www.cijfersoverkanker.nl). Important risk factors for esophageal adenocarcinoma (EAC) are obesity and reflux (31) while Helicobacter pylori infection and family history of gastric cancer are two important risk factors for gastric cancer (32, 33). Like pancreatic cancer, most patients with resectable esophageal and gastric cancer cannot be cured with surgery alone. Preoperative CRT with carboplatin and paclitaxel compared to surgery alone improved the ten-year survival rate of patients with resectable esophageal or esophagogastric-junction cancer from 25 to 38 percent (HR 0.7) (34, 35) and this is currently the standard treatment. Recently the CheckMate 577 trial showed adjuvant immunotherapy with nivolumab to be associated with a longer disease free survival (DFS) compared to placebo (22,4 vs. 11, 0 months, HR: 0,69; $p < 0,001$) (36). Treatment with CRT without resection can be a good treatment option to improve survival for patients in a good clinical condition who present with locally advanced irresectable esophageal or esophagogastric-junction cancer (37). Patients with resectable gastric cancer do also benefit of systemic treatment before resection. In the MAGIC trial 3 preoperative and 3 postoperative cycles of epirubicin, cisplatin, and 5-FU (ECF) in combination with resection vs. resection alone resulted in a better OS (HR 0.75; 5 year OS: 36 versus 23 percent) (38). Perioperative chemotherapy with the docetaxel-based triplet perioperative FLOT (5-FU plus leucovorin, oxaliplatin and docetaxel) chemotherapy schedule further improved OS to a median 50 months vs 35 months (HR 0.77) with perioperative ECF/ECX (epirubicin, cisplatin and 5-FU or capecitabine) (39) and this is therefore the current standard therapeutic approach for patients with resectable gastric cancer. Palliative treatment for advanced esophageal and gastric cancer frequently consists of combination chemotherapy and/or radiotherapy. The goals of palliative systemic chemotherapy are survival benefit and relief of cancer-related symptoms (31, 40). First-line treatment with oxaliplatin and capecitabine is presently the most commonly employed approach for patients with advanced or metastatic

esophageal cancer (41, 42). Systemic treatment options for patients with advanced esophageal and gastric cancer are further described in detail in the summarizing discussion of this thesis.

In **chapter 4** we describe the results of a multicenter randomized open label phase 2 trial aiming to improve the RR of palliative first-line chemotherapy for patients with advanced EC and GC by adding folic acid and vitamin B12 to the at the time commonly used schedule of cisplatin in combination with gemcitabine (43, 44). The rationale for adding folic acid and vitamin B12 was based on the efficacy and toxicity benefits that were observed in a phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant mesothelioma(45). Preclinical evidence demonstrated that differences in the folate environment resulted in a different sensitivity of human cancer cell lines to cisplatin (46, 47).

In **chapter 4**, we first investigated the sensitivity of adenocarcinoma cell lines for cisplatin under high and low folate conditions. The observation that adenocarcinoma cells grown under high folate conditions appeared to be more sensitive to the effects of cisplatin and the intracellular platinum accumulation was higher in these cells, provided further preclinical support for the clinical study evaluating the addition of folic acid and vitamin B12 suppletion to first-line palliative cisplatin and gemcitabine in patients with advanced esophagogastric cancer as described in **chapter 4**.

In the current landscape of increased treatment options and increasing medicine costs, patient selection for the right treatment is very important to avoid unnecessary exposure of patients to toxic treatments and to enhance efficient use of financial resources. Predictive and prognostic biomarkers are important to select patients that may benefit most from treatment (48). MicroRNAs (miRNAs) are extensively studied as potential biomarkers for different tumor types. miRNAs are endogenous, evolutionarily conserved, small non-coding RNAs, that regulate post-transcriptional gene expression through complementary binding to the 3'untranslated regions (3'UTRs) of target messenger RNA (mRNA) genes (49). A landmark study in 2005 demonstrated that tissue miRNA expression profiles have the ability to classify different types of human cancer, highlighting the potential of miRNA profiling as tool for cancer diagnosis (50). It appeared that miRNAs that target tumor suppressor genes function as oncogenes while miRNAs that target oncogenes exert a tumor suppressor function in different cancer types (51). This is further explained in figure 2.

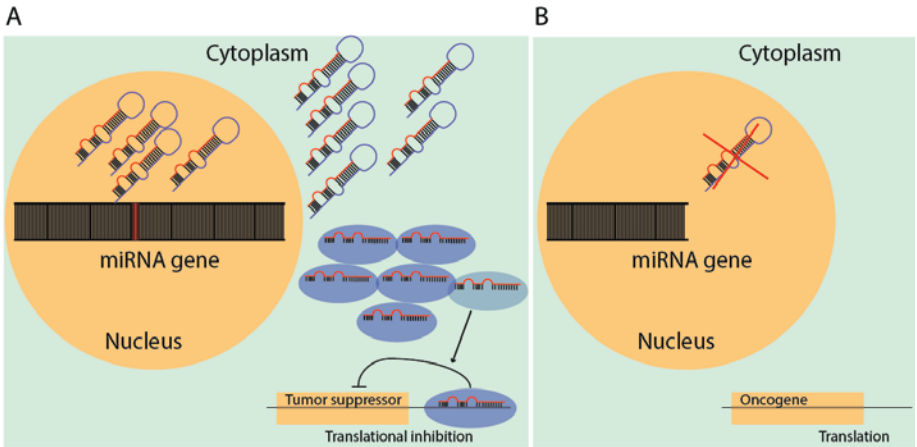

Figure 2. The regulation of tumor suppressor genes and oncogenes by miRNAs. **A** Regulation of tumor suppressor genes by miRNAs. miRNA levels can be increased due to a mutation (in red). When these overexpressed miRNAs are processed into a miRNA/RISC complex as described in **Fig. 1**, and target a specific tumor suppressor gene, these cells can become cancer cells. **B** Regulation of oncogenes by miRNAs. miRNA levels can be decreased due to for example a deletion in a miRNA gene. This can lead to overexpression of oncogenes, normally inhibited by miRNAs, resulting in increased proliferation and tumor formation (image with permission of dr D. Poel).

miRNAs remain stable under different conditions, such as changes in temperature and pH. This stability allows miRNAs to remain detectable and quantifiable even in tissue that has been stored over extended periods (52). Innovative technologies now allow for the quantification of expression levels of numerous miRNAs from small tissue samples, which has stimulated further miRNA research (53, 54). Additionally, in 2008, circulating miRNAs (ci-miRNAs) released from tumor tissue were identified in blood using real-time polymerase chain reaction (RT-PCR). These ci-miRNAs were surprisingly stable in the blood circulation and therefore proposed as a new possible liquid biomarker for cancer (55, 56). In 2012, droplet digital PCR (ddPCR) revolutionized miRNA measurement. By partitioning samples into thousands of droplets for PCR reactions, ddPCR offers absolute miRNA copy counts, ensuring greater sensitivity compared to RT-qPCR (57). The definite role of miRNAs in EC and GC is unclear due to conflicting study outcomes (58-60) and limited data are available about the function of miRNAs in plasma of patients treated with palliative chemotherapy for EC and GC. We describe in **chapter 5** a study that focusses on the prognostic and predictive value of ci-miRNAs using droplet digital PCR (ddPCR) in plasma samples of patients with advanced EC and GC that were treated in the trial described in **chapter 4**.

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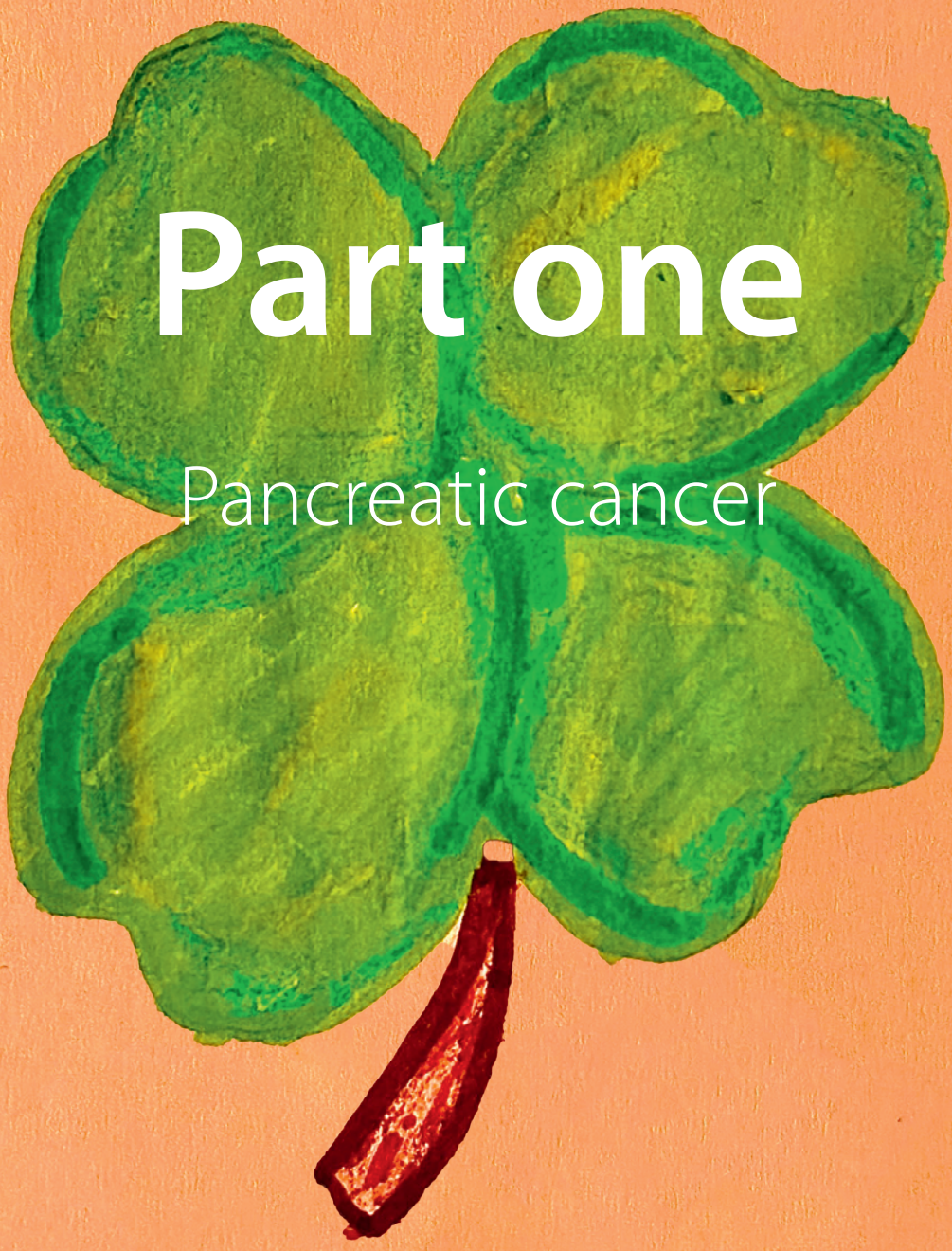

Part one

Pancreatic cancer

Phase I clinical trial to determine the feasibility and maximum tolerated dose of panitumumab to standard gemcitabine-based chemoradiation in locally advanced pancreatic cancer

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Annette A. van Zweeden[1], Hans J.van der Vliet[1], Johanna W. Wilmink[2], Martijn R. Meijerink[3], Otto W.M. Meijer[4], Anna M.E. Bruynzeel[4], Geertjan van Tienhoven[5], Elisa Giovannetti[1], Geert Kazemier[6], Maarten A.J.M. Jacobs[7], and Henk M.W. Verheul[1]

[1] Department of Medical Oncology, VU University Medical Center, Amsterdam, the Netherlands. [2] Department of Medical Oncology, Academic Medical Center, Amsterdam, the Netherlands. [3] Department of Radiology and Nuclear Medicine, VU University Medical Center, Amsterdam, the Netherlands. [4] Department of Radiation Oncology, VU University Medical Center, Amsterdam, the Netherlands. [5] Department of Radiation Oncology, Academic Medical Center, Amsterdam, the Netherlands. [6] Department of Surgery, VU University Medical Center, Amsterdam, the Netherlands. [7] Department of Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, the Netherlands.

Corresponding Author: Henk Verheul, VU University Medical Center, De Boelelaan 1117, Amsterdam 1081 HV, the Netherlands. Phone: 31-20-4444321; Fax: 31- 20-4444355; E-mail: h.verheul@vumc.nl

Abstract

Purpose: Epidermal growth factor receptor (EGFR) inhibitors may improve both the therapeutic efficacy of radiotherapy and the radiosensitizing activity of gemcitabine. Based on this rationale and the nonoverlapping toxicity profiles of gemcitabine and the monoclonal EGFR antibody panitumumab, we designed a phase I trial to investigate the maximum-tolerated dose (MTD), safety, and activity of panitumumab added to gemcitabine-based chemoradiotherapy (CRT) in patients with locally advanced pancreatic cancer (LAPC).

Experimental Design: Patients with LAPC and WHO performance status 0 to 1 were treated with weekly panitumumab at four dose levels (1–2.5 mg/kg), combined with weekly gemcitabine 300 mg/m2 and radiotherapy (50.4 Gy in 28 fractions) for 6 weeks, followed by gemcitabine 1,000 mg/m2 weekly for 3 weeks every 4 weeks until disease progression or unacceptable toxicity. Each cohort was monitored during the combination therapy to establish dose limiting toxicity. Tumour evaluation was per- formed after CRT and during gemcitabine monotherapy.

Results: Fourteen patients were enrolled; 14 were evaluable for toxicity and 13 for response. The MTD for panitumumab was 1.5 mg/kg. Three of the 6 patients, treated at MTD, experienced grade 3 adverse events during the combination therapy; neutropenia (n = 2; 33%), fatigue (n = 1; 17%), nausea (n = 1; 17%), and vomiting (n = 1; 17%). Partial response was achieved by 3 patients (23%), 1 in each dose cohort. Median progression free survival of the three cohorts together was 8.9 months.

Conclusions: The addition of panitumumab to gemcitabine- based chemoradiotherapy in LAPC has manageable toxicity and potential clinical efficacy.

Translational Relevance

Epidermal growth factor receptor (EGFR) expression is high in pancreatic cancer. Preclinical evidence indicates that EGFR pathway inhibition improves antitumor efficacy of radiotherapy independent of the K-RAS mutation status of a tumor. Preclinical in vivo studies have also shown that EGFR inhibition enhances the radiosensitizing activity of gemcitabine. Panitumumab, a fully human anti-EGFR monoclonal anti- body, increased the antitumor efficacy of gemcitabine in a pancreatic tumor model. In this phase 1 trial, we investigated the maximum-tolerated dose (MTD), safety, and activity of panitumumab when combined to gemcitabine-based chemoradiotherapy in patients with locally advanced pancreatic cancer (LAPC). The addition of panitumumab to gemcitabine- based chemoradiotherapy in LAPC has manageable toxicity and showed first evidence of clinical efficacy. These observations support the further evaluation of this combination in a phase II study.

Introduction

In the Western world, approximately 5% of cancer mortality is due to pancreatic ductal adenocarcinoma (PDAC). At presentation, only 10% to 20% of patients with PDAC have localized disease that can be considered for resection. The remaining patients cannot be cured with resection due to locally advanced pancreatic cancer (LAPC; approximately 35%) or metastatic disease. Treatment of LAPC is extremely challenging. Despite recent advances in the treatment of metastatic pancreatic cancer using combination chemotherapy (CHT) regimens, including oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX; ref. 1), or gemcitabine–paclitaxel protein bound (2), not much progress has been made in the treatment of LAPC in the last decade. Because both metastatic spread and locoregional disease progression are of major concern, combinations of systemic CHT and radiotherapy (RT) are often used. Numerous chemoradiotherapy (CRT) studies have been performed, attempting to postpone disease progression and also to increase the possibility of resection (3, 4). Results of these CRT regimens are comparable, and do not consistently show benefit of combination treatment or from CHT alone (5). However, a recent meta-analysis including 15 randomized trials in which CRT with RT or CHT alone were compared for LAPC showed superiority of CRT in 6- and 12- month survival at the expense of more toxicity (6). Survival at 18 months was not significantly different. Ongoing developments include the use of CRT regimens based upon full dose CHT with added radiation, rather than the other way round (7, 8) or to add additional drugs to improve outcome of CRT (9). Although CRT is considered as standard treatment for LAPC, improvement in efficacy and reduction of toxicity is urgently needed.

The epidermal growth factor receptor (EGFR; also known as ErbB-1 and HER1 in humans) signalling cascade plays an important role in the biology of various malignancies, including pancreatic cancer. Human pancreatic cancer cells overexpress EGFR and its known ligands (10, 11), which is correlated with rapidly progressive disease (12). EGFR inhibition by the addition of erlotinib, a tyrosine kinase inhibitor, to gemcitabine is associated with a modest survival benefit in PDAC (13). In colorectal cancer, the clinical efficacy of antibodies against EGFR is restricted to patients with wild-type RAS tumors (14, 15). PDAC is known for its high K-RAS mutation phenotype (>90%) and harbors the highest reported incidence of RAS mutations among all human cancers (16). These mutations rarely

affect H-RAS or N-RAS and concentrate almost exclusively on the K-RAS locus, with reports of mutation rates up to 95% (17).

Interestingly, several studies have shown that EGFR pathway inhibition can improve the antitumor efficacy of RT independent of the K-RAS mutation status of a tumor (18, 19). Furthermore, preclinical in vivo studies indicated that the radiosensitizing activity of gemcitabine may be enhanced by specific EGFR inhibition (20) and also showed, in a pancreatic tumor model, that treatment with panitumumab, a fully human anti-EGFR monoclonal antibody (mAb), enhanced the antitumor efficacy of gemcitabine monotherapy (61% vs. 38% growth inhibition; ref. 21). Panitumumab is generally well tolerated. Skin toxicity, hypomagnesaemia, and diarrhoea are the most common toxicities observed (22). Based on these promising preclinical data in favour of combining EGFR pathway inhibition (independent of K-RAS mutational status) with both gemcitabine and radiation therapy and the nonoverlapping toxicity profiles of gemcitabine and panitumumab, we designed a phase I/II feasibility trial evaluating the addition of panitumumab to gemcitabine-based CRT in patients with LAPC. Here, we report the phase I feasibility part of this study. The primary endpoint was to determine the maximum tolerated dose (MTD) of panitumumab to be used in combination with gemcitabine-based CRT in patients with LAPC. Secondary endpoints included early signs of clinical activity of the study treatment, clinical response rate, progression free survival (PFS), and overall survival (OS).

Patients and Methods

Eligibility

Adult patients with untreated LAPC were eligible for this phase I trial. Encasement >270 of the superior mesenteric or portal vein or >90 tumor contact with the superior mesenteric artery, celiac trunk, or common hepatic artery in the absence of distant metastasis was considered in a multidisciplinary team as LAPC. Histologic or cytologic confirmation of pancreatic cancer was required. Patients with imminent bowel obstruction, active bleeding, uncontrolled infection, or a second current malignant disease (except basal cell carcinoma of the skin) were not eligible. Inclusion criteria also included measurable or evaluable disease as defined by response evaluation criteria in solid tumors (RECIST) 1.1 criteria, an

Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) of 0 or 1, adequate hematological, hepatic (2.5 upper limit of normal, ULN) and renal function (estimated glomerular filtration rate >50 mL/min), normal calcium and magnesium levels. Patients with a history of allergic reactions to antibody treatment, impossibility of adequate radiation therapy, for example, due to tumor size, and patient suffering from any serious concomitant systemic disorders incompatible with the clinical study were considered not eligible. The institutional Medical Ethical board of the two participating centers, VU University Medical Center and the Academic Medical Center (both in Amsterdam, the Netherlands), approved the conduction of the trial (ClinicalTrials.gov Identifier: NCT01175733), which was in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients prior to inclusion into the study.

Study treatment

The treatment scheme is summarized in Fig. 1. The study was designed as 3 þ 3 dose escalation clinical trial (23, 24). Panitumumab was administered weekly for 6 weeks by intravenous infusion in different dose levels per cohort (1, 1.5, 2, and 2.5 mg/kg) during gemcitabine-based CRT. Gemcitabine was administered weekly by intravenous infusion at a dose of 300 mg/m2 during RT in the first 6 weeks, followed by a dose of 1,000 mg/m2 weekly for 3 weeks every 4 weeks from day 50 until disease progression or unacceptable toxicity and otherwise continued for a maximum period of 1 year. The RT schedule consisted of a dose of 1.8 Gy per fraction for 28 days (total dose of 50.4 Gy) between days 1 and 38, on days 3 to 5 of the first week and on day 1 to 5 in the five consecutive weeks. The planning target volume for the radiation treatment consisted of all gross tumor on a 4D-CT scan including regional enlarged lymph nodes, enlarged with a 1.0-cm margin. The craniocaudal margin was extended with an extra 1 cm, when a 3D-CT scan was used. Typically, a multiple coplanar field technique or a volumetric modulated arc technique was used for treatment planning. Depending on the radiation oncology center (VUmc or AMC), either spinal column imaging or intratumoral fiducial markers were used for daily set-up. Follow-up lasted until death of the patient. In case of progressive disease, the choice of offering further treatment with palliative CHT was at the discretion of the treating physician.

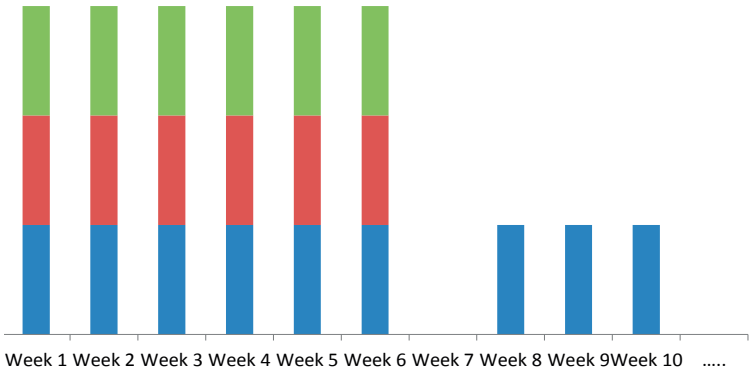

Figure 1. Treatment schedule for patients with LAPC.

Toxicity evaluation, dose escalation rules, and response assessment

Dose limiting toxicity (DLT) and adverse events (AE) were graded by the NCI Common Terminology Criteria of Adverse Events (CTC-AE) grading system version 3.0 (25). Patients were continuously monitored for toxicity. Patients were enrolled per dose level in cohorts of 3. At the final dose level, a minimum of 6 patients were treated to determine the MTD, defined as less than 2 patients out of 6 with a DLT. DLTs were defined as any of the following hematologic events during the first 43 days from start of combination treatment and attributable to combination therapy: febrile neutropenia, neutropenic infection, grade 4 neutropenia lasting over 7 days, grade 3 thrombocytopenia for >7 days, and grade 4 thrombocytopenia. Non-hematological DLTs included grade 3 nausea, vomiting, or diarrhea despite optimal medical support, grade 3 fatigue persisting >7 days, or grade 4 fatigue. Any other grade 3 AE (except alopecia), failure to recover from related toxicities to grade 1 or baseline severity (or grade 2 at investigator and sponsor discretion) after delaying the next cycle up to 7 days and failure to complete the first 6 weeks of treatment (75% of planned dose of RT, gemcitabine, and panitumumab) were also considered as a DLT. Mucositis, diarrhea, dermatitis, and rash were considered as specific panitumumab-related toxicities. The use of prophylactic treatment for skin toxicities was allowed. All patients underwent a baseline CT scan and an efficacy evaluation CT after chemoradiation, during gemcitabine monotherapy at 3, 5, 7, and 9 months and every 3 months thereafter until progressive disease. The RECIST version 1.1 (26) were used for response assessment. Time to progression (TTP), mentioned in the study protocol as secondary endpoint, was defined as the time from registration in the study to progression or death, whichever came

first. TTP is further described in this study as PFS. Measurements of CA19.9 (U/mL) were performed before and during treatment. CA19.9 response was defined as a decrease in CA19.9 concentration of at least 50% from the baseline concentration to the lowest value (nadir) measured during the study.

Results

Patient characteristics

Twenty patients were registered in this study. Five patients were considered ineligible, because of either metastatic disease (n = 3), elevated liver enzymes (n = 1), and patient withdrawal before start of treatment (n = 1). Between July 2010 and November 2013, treatment was initiated in 15 patients. One patient in the 1.5 mg/kg cohort withdrew her consent after 2 weeks and was therefore considered not evaluable. The clinicopathologic characteristics of the 14 treated patients are listed in Table 1.

Table 1. Clinicopathologic characteristics for 14 patients receiving chemoradiation with panitumumab for LAPC

Characteristic	Patients, *n* (*N* =14)	%
Age (years at the start of the treatment)	63 (46–77)	
Sex		
Male	11	79
Female	3	21
Histology/cytology		
Adenocarcinoma	13	93
Other: neuro-endocrine tumor	1	7
Diagnosis based on Cytology	4	
Histology	10	
T-stage		
T3	1	7
T4	13	93
N stage		
N0	14	100
N1	0	0
ECOG PS at start		
0	9	64
1	5	36
Ca19,9 prior to therapy, U/mL		
Median	103	
Range	5–1,499	

DLT and maximum-tolerated and safe dose

No DLTs were observed in the first 3 patients in the first and second dose level (1 and 1.5 mg/kg panitumumab). Two of the 5 patients treated in the third dose level (2 mg/kg panitumumab) experienced a DLT and therefore this dose level was considered non-tolerable. One of these patients fulfilled the DLT criteria, because of nausea grade 3 despite optimal medical support, including hospitalization. This was reported as a serious adverse event (SAE) related to panitumumab in combination with gemcitabine and RT. The other patient experienced a DLT based on failure to complete the first 6 weeks of treatment as defined by the protocol, due to multiple grade 1 to 2 toxicities. The next 3 patients were enrolled in the second dose level (1.5 mg/kg) and none had a DLT, providing the MTD as defined in the protocol. Toxicity was manageable at a panitumumab dose of 1.5 mg/kg (MTD). All 6 patients in this dose cohort experienced some grade of nausea and vomiting and 3 of the 6 patients experienced one or more (possibly) treatment-related grade 3 AEs during the first 43 days of treatment. One patient experienced neutropenia, the second patient experienced neutropenia, nausea, and vomiting, and the third patient experienced fatigue. These AEs were manage- able and resolved within 4 days (nausea and vomiting), 5/6 days (neutropenia), and 6 days (fatigue). No grade 4 AEs were observed at the MTD. All AEs during CRT with panitumumab are listed in Table 2. Rash, nausea, vomiting, weight loss, taste alteration, diarrhea, paronychia, and dermatitis are considered as (possible) panitumumab-related AEs. Four patients experienced hypomagnesaemia grade 1, 3 patients only during CRT, 1 patient also during the first 2 months of gemcitabine monotherapy. A total of 12 SAEs were reported in 8 of the 14 study participants, which were evaluable for toxicity. Only one SAE was related to the study treatment. AEs reported after the CRT period (first 43 days) of the study are listed in Supplementary Table S1. No unexpected AEs were reported during gemcitabine monotherapy.

Table 2. Adverse events during chemoradiation with panitumumab (maximum grade of each AE per patient) during the chemoradiation with panitumumab (first 43 days)

AE, *n* (%)	Cohort 1.0 mg/kg (*n* =3)		Cohort 1.5 mg/kg (*n* =6)		Cohort 2.0 mg/kg (*n* =5)	
	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
Blood/bone marrow	—		2 (33)			
Thrombocytopenia		—		—	—	—
Neutropenia	—	—	2 (33)	2 (33)	—	1 (20)

Table 2. continued)

AE, *n* (%)	Cohort 1.0 mg/kg (*n* =3)		Cohort 1.5 mg/kg (*n* =6)		Cohort 2.0 mg/kg (*n* =5)	
	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
Constitutional symptoms	2 (67)	—	4 (67)	1 (17)	2 (40)	—
Fatique (asthenia, lethargy, malaise)						
Weary legs	—	—	1 (17)	—	—	—
Fever	1 (33)	—	2 (33)	—	—	—
Weight loss	—	—	1 (17)	—	2 (40)	—
Insomnia	—	—	—	—	1 (20)	—
Neurology	—	—	—	—	1 (20)	—
Dizziness when getting up						
Neuropathy	—	—	1 (17)	—	—	—
Syndromes	—	—	1 (17)	—	—	—
Flu like syndrome						
Pulmonary/upper respiratory	—	—	1 (17)	—	—	—
Cough						
Hiccups	—	—	1 (17)	—	1 (20)	—
Gastrointestinal	1 (33)	—	5 (83)	1 (17)	3 (60)	1 (20)
Nausea						
Vomiting	—	—	5 (83)	1 (17)	4 (80)	—
Stomach complaints	1 (33)	—	1 (17)	—	—	—
Anorexia	1 (33)	—	4 (67)	—	4 (80)	—
Flatulence	—	—	1 (17)	—	—	—
Constipation	—	—	3 (50)	—	—	—
Taste alteration	—	—	1 (17)	—	—	—
Diarrhea	—	—	1 (17)	—	1 (20)	—
Pyrosis	—	—	—	—	1 (20)	—
Pain	1 (33)	—	—	—	—	—
Glossodynia						
Tumor pain	—	—	1 (17)	—	—	—
Pain	1 (33)	—	—	—	1 (20)	—
Abdominal pain	1 (33)	—	2 (33)	—	—	—
Dermatology/skin	—	—	1 (17)	—	—	—
Dry skin						
Acneiform rash	2 (67)	—	2 (33)	—	3 (60)	—
Paronychia big toe	1 (33)	—	—	—	—	—
Node in groin region	1 (33)	—	—	—	—	—
Rash	1 (33)	—	2 (33)	—	1 (20)	—
Red skin (face)	—	—	1 (17)	—	—	—
Pruritus/itching	—	—	—	—	1 (20)	—
Erythema (face/groin/ abdomen)	—	—	1 (17)	—	—	—
Renal/genitourinary	—	—	1 (17)	—	—	—
Dysuria						

Table 2. continued)

AE, *n* (%)	Cohort 1.0 mg/kg (*n* =3)		Cohort 1.5 mg/kg (*n* =6)		Cohort 2.0 mg/kg (*n* =5)	
	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
Hemorrhage/bleeding	—	—	—	—	1 (20)	—
Epistaxis						
Gastrointestinal	—	—	—	—	1 (20)	—
Metabolic/laboratory	—	—	1 (17)	—	—	—
Elevated transaminases						
Hypomagnesemia	—	—	—	—	1 (20)	—
Infection	1 (33)	—	—	—	—	—
Cellulitis						
Urinary tract infection	—	—	1 (17)	—	—	—

Response, PFS, and OS

One patient in the 1 mg/kg cohort was diagnosed with LAPC based on cytology, but when he developed metastatic disease, histology revealed a pancreatic neuroendocrine tumor. Therefore, this patient was evaluated for toxicity, but not for tumor response, PFS, and OS. In Table 3, the median PFS and OS of the patients treated in this study are shown. The median PFS of all cohorts together is 8.9 months (range, 3.5–23), including 1 patient who had stable disease under study treatment according to protocol after 10.9 months of follow-up. The median PFS in the MTD cohort is 11.8 months (range, 3.7–23). Two of the 13 patients evaluable for response were still alive at the time of evaluation; both were treated in the MTD cohort. The median OS in all cohorts together is 12.3 months (range, 4.0–30.1 months), including the 2 patients who were alive at the last evaluation (respectively 10.9 and 20.1 months from start until last evaluation). The median OS in the MTD cohort is 17.0 months (range, 10.9–25.8), including 2 patients who were alive at the last evaluation. Three patients achieved a partial response (23%), whereas all other patients had stable disease as best response. Median duration of gemcitabine monotherapy after chemoradiation is 3.9 months for all patients and 4.3 months for the patients treated in the MTD cohort, including the last patient still under study treatment after 10.9 months follow-up. CA19.9 response was reached by 8 of the 13 (61.5%) for response evaluable patients, 5 of them were treated in the MTD cohort. These 8 patients had stable disease when reaching CA19.9 response and a median PFS and OS of respectively 9.9 and 13.0 months.

Table 3. Median PFS and OS for each dose cohort

	1 mg/kg	1,5 mg/kg	2,0 mg/kg
n	3*	6**	5
Median PFS (per cohort)	11.0	11.8	4.3
median OS (per cohort)	19.5	17.0	9.1

*One patient in the 1 mg/kg cohort is not assessable for tumor response, PFS and OS
**In the 1,5 mg/kg cohort 1 patients were progression free at evaluation and 2 patients were alive at evaluation.

Discussion

Here, we report the results of a phase I study designed to determine the safety, tolerability, and potential clinical efficacy of the anti-EGFR mAb panitumumab added to standard gemcitabine-based CRT in patients with LAPC. The MTD of panitumumab was determined to be 1.5 mg/kg. We concluded that adding panitumumab to gemcitabine-based CRT is feasible with considerable, but manageable toxicity, as expected based on the non- overlapping toxicity profile of CRT and panitumumab. No differences in performance status, nodal status (Table 1), comorbidity, intensity of RT (tumorsize and radiation fields were equal between the cohorts; Supplementary Table S2), or dosing of gemcitabine by itself could explain the observed DLTs in cohort three compared with the other two cohorts and were therefore most likely caused by the addition of a higher dose of panitumumab to the combination treatment. Major toxicities potentially related to the addition of panitumumab were nausea, vomiting, neutropenia, fatigue, and anorexia, whereas acneiform rash was considered to be definitively related. These toxicities, apart from skin toxicity (22), are not common for treatment with anti-EGFR mAb treatment, and are likely to be predominately caused by the combination with gemcitabine-based CRT (8, 27). In addition, patients with LAPC often suffer from fatigue and gastrointestinal symptoms such as nausea and vomiting, even without treatment. Because of these common gastrointestinal problems and the small number of patients per cohort in this study, we cannot rule out that establishing the MTD might be influenced by mild differences of gastrointestinal or even other complaints in the three cohorts before start of treatment. How- ever, these differences were not clinically recognized despite intensive observation and there is no evidence of objective measurable differences that may have influenced

gastrointestinal complaints between the cohorts except for the difference in combination treatment (e.g., higher dose of panitumumab in cohort 3 compared with cohorts 1 and 2).

Whether this combination treatment is of clinical benefit needs to be studied in the phase II part of this study. The first preliminary results on this small study population with a median PFS and OS of respectively 11.8 and 17.0 months in the MTD cohort suggest some efficacy. However, a true comparison of efficacy related to the different dose cohorts is not possible due to the small numbers of patients. OS and PFS were relatively short for patients treated in the high-dose cohort, while having comparable base line characteristics compared with patients in the MTD cohort (respectively 60% vs. 50% WHO PS 0 and respectively 90% vs. 83% elevated baseline Ca 19.9). The distribution of T4 tumors was also equal between the different cohorts. Multiple studies included targeted therapy. Different CHT schedules and RT modalities report variable outcomes without a clear advantage of one treatment schedule. Our previous reports on chemoradiation for LAPC revealed a median survival of 10 months for the combination of gemcitabine 300 mg/m2 weekly with RT (24 Gy in three consecutive weekly fractions of 8 Gy) (27) and for the combination of UFT 300 mg/m2 daily, leucovorin 30 mg and celecoxib 800 mg daily for 28 days concomitant with RT (20 x 2.5Gy) (28). In the present trial, radiation and CHT seemed to be better tolerated compared with our previous studies. The day to day position variation (29) of pancreatic tumors and their intrafraction motion (30) due to breathing pose are challenging concerns in RT of LAPC. Currently, we tackle these issues by performing image guided radiation therapy (IGRT), using gold fiducial markers (31).

Our study is the first clinical trial in which panitumumab is combined with gemcitabine-based CRT in LAPC. Previously, the combination of panitumumab 6 mg/kg on days 1, 15, and 29 in combination with 5FU/capecitabine-based CRT followed by gemcitabine and panitumumab followed by maintenance panitumumab for 6 months in LAPC was reported at ASCO 2012 (32). At a median follow-up of 12.3 months, median OS and PFS were 12.1 and 7.4 months, respectively. AEs (67% grade 3 and 20% grade 4), especially during the chemo-RT portion, were considerable and affected administration of subsequent systemic maintenance therapy. The observed toxicities in the current dose finding study suggest that the panitumumab dose was too high, at least during CRT. Other anti-

EGFR mAbs such as cetuximab have also been evaluated as a treatment strategy for LAPC. Panitumumab and cetuximab both target the EGFR but they differ in their isotype and they might differ in their mechanism of action. An OS of 7.5 months was reported for RT in combination with single-agent cetuximab. This OS is less than for most CRT trials in LAPC but the toxicity was also very moderate (33). Crane and colleagues demonstrated a favorable OS of 19.2 months of cetuximab in combination with induction CHT (gemcitabine and oxaliplatin) in LAPC followed by capecitabine based CRT (50.4 Gy) in combination with cetuximab (34). The toxicity was comparable to this study, except an increased incidence of sensory neuropathy, which is associated with oxaliplatin.

Other targeted therapies such as the VEGF mAb bevacizumab, the tyrosine kinase inhibitor sorafenib, and the cyclooxygenase-2 inhibitor, celecoxib, have also been investigated in combination with chemoradiation, but did not result in a significant improvement in OS (28, 35, 36). The combination of erlotinib, bevacizumab, and external beam radiation therapy without CHT in a phase I trial was reasonably well tolerated as presented at ASCO GI in 2011 (37). CRT trials that have been performed studying the added effect of targeted therapy are summarized in Table 4.

Novel local therapies such as radiofrequency ablation (RFA) and irreversible electroporation (IRE) are used and studied in increasing frequency in the treatment of LAPC (38). RFA is a thermal local therapy based on high-frequency electrical currents. Variable outcomes of the efficacy of RFA are described in small nonrandomized trials (39, 40). IRE is a promising nonthermal ablative technique using direct current, which irreversibly damages the cell's homeostatic mechanism, causing apoptosis. Two series were reported of IRE in PDAC with promising results and manageable toxicity (41, 42). Stereotactic body RT (SBRT) is a recent advancement that allows for the precise delivery of a large ablative radiation dose to the tumor in one to five fractions. A total dose between 24 and 36 Gy in one to five fractions has been reported (43–45). SBRT could be delivered quickly and effectively in patients with LAPC with acceptable side effects and minimal interference with gemcitabine CHT. An advantage of IRE and stereotactic RT over RFA is that they can be used for tumors in close proximity to large vessels without risk of vascular trauma or a reduced effect of RFA due to the heat sink effect (46). No randomized studies of RFA, IRE, or stereotactic radiation have been published. These local treatment modalities in this setting are of interest because

they are presumably better tolerated than CRT. Yet the efficacy of these approaches compared with the efficacy of CRT for LAPC has to be established. The above local treatment possibilities illustrate the challenging options for patients with LAPC.

In conclusion, we report that the use of panitumumab at a MTD of 1.5 mg/kg can be safely added to gemcitabine-based CRT in patients with LAPC. The observed PFS and OS rates suggest some efficacy. These observations support the further evaluation of this combination in a phase II study along with the search of predictive biomarkers to allow future selection of patients with an increased chance of experiencing clinical benefit from this type of combination therapy.

Table 4. Trials of CRT + targeted therapies for pancreatic cancer

Characteristics of CRT trials in combination with targeted therapy				
Ref.	Study treatment	Patients, *n*	Median TTP (mo)	Median survival (mo)
This study, 2015	RT(50.4 Gy)+GEM+PAN-GEM	13	8.9 MTD cohort: 11.8	12.3 MTD cohort: 17.0
Kim, 2012 (abstr) (32)	PAN+5FU/CAP+RT (50.4 Gy)-GEM+PAN-PAN	51	7.4	12.1
Rembielak et al. (33)	CET+RT (50.4 Gy)	21	5.1	7.5
Crane et al. (34)	CET+GEM+OX- CAP+CET+RT (50.4 Gy)	69	12.5	19.2
Crane et al. (35)	CAP+RT (50.4 Gy)+BEV-GEM+BEV.	82	8.9	11.9
Chiorean et al. (36)	SOR + GEM + RT (50 Gy)-GEM	25	10.6	11.4
Morak et al. (28)	UFT + L + C + RT (50 Gy)	83	6.9	10.6
Czito et al. (37)	E + BEV + RT (50.4 Gy)	9	[a]	[a]

Abbreviations: BEV, bevacizumab; C, celecoxib; CAP, capecitabine; CET, cetuximab; GEM, gemcitabine; E, Erlotinib; L, leucovorin; OX, oxaliplatin; PAN, panitumumab; SOR, sorafenib; UFT, Uracil/Tegafur. [a]Med TTP and OS not mentioned in abstract, study is not published

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Tables

Supplementary Table 1

Suplementary Table 1. Adverse events during chemoradiation with panitumumab, according to CTCAE criteria (after 43 days)

	Cohort 1.0 mg/kg (n=3)		Cohort 1.5 mg/kg (n=6)		Cohort 2.0 mg/kg (n=5)	
AE, n (%)	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4	Grade 1/2	Grade 3/4
BLOOD/ BONE MARROW						
Thrombocytopenia	-	-	-	2 (33)	1 (20)	-
Anemia	2 (67)	-	2 (33)	-	-	-
Neutropenia	-	1 (33)	-	3 (50)	-	-
METABOLIC/LABORATORY						
Creatinine increase	1 (33)	-	-	-	-	-
CONSTITUTIONAL SYMPTOMS						
Fatique	1 (33)	-	5 (83)	1 (17)	-	-
Fever	3 (100)	-	1 (17)	-	1 (20)	-
NEUROLOGY						
Neuropathy	1 (33)	-	-	-	-	-
SYNDROMES						
Flu like syndrome	1 (33)	-	-	-	2 (40)	-
PULMONARY/UPPER RESPIRATORY						
Dyspnea	1 (33)	-	-	-	-	-
LYMPHATICS						
Edema	2 (67)		-	-	-	-
GASTROINTESTINAL						
Nausea	2 (67)	-	2 (33)	-	1 (20)	-
Vomiting	-	-	3 (50)	-	-	-
Anorexia	1 (33)	-	4 (67)	-	-	-
Ascites (non malignant)	-	-	-	1 (17)	-	-
Flatulence	1 (33)	-	-	-	-	-
HEMORRHAGE/BLEEDING						
Gastrointestinal bleeding	-	-	-	1 (17)	-	-
PAIN						
Headache	1 (33)	-	1 (17)	-	-	-
Pain feet	-	-	1 (17)	-	1 (20)	-
Muscle pain	-	-	1 (17)	-	-	-
DERMATOLOGY/SKIN						
Dry blister	-	-	1 (17)	-	-	-
Acneiform rash	1 (33)	-	1 (17)	-	3 (60)	-
Hair loss/ alopecia	-	-	1 (17)	-	-	-
INFECTION						
Urinary tract infection	-	-	1 (17)	-	-	-
Pneumonia	-	-	1 (17)	-	-	-

Supplementary Table 2

Cohort	Mean (median) tumor size (mm)*	Standard error of mean (mm)
1 mg/kg	37 (33)	11
1,5 mg/kg	43 (38)	8
2 mg/kg	43 (36)	16

*Longest diameter of measurable primary tumor

Reintroduction of palliative intent FOLFIRINOX chemotherapy in a real world pancreatic cancer cohort

submitted

Annette A. van Zweeden, MD[1,2], Lydia.G.M. van der Geest, PhD[1,3], Esther N. Pijnappel, MD, PhD[1], Judith de Vos-Geelen, MD, PhD, assistant professor[4], Jeanin E. van Hooft, MD, PhD, professor[5], Martijn W.J. Stommel MD, PhD[6], Gijs F. de Wit, MD[7], Henk M.W. Verheul, MD, PhD, professor[8], Hanneke W.M. van Laarhoven, MD, PhD, professor[1,9], Hans J. van der Vliet, MD, PhD, professor[1,9,10] and Johanna W. Wilmink, MD, PhD, professor[1,9*] on behalf of the Dutch Pancreatic Cancer Group

[1] Amsterdam UMC, University of Amsterdam, Department of Medical Oncology, Amsterdam, The Netherlands [2] Amstelland Hospital, Department of Internal Medicine, Amstelveen, The Netherlands [3] Netherlands Comprehensive Cancer Organization (IKNL), Department of Research and Development, Utrecht, The Netherlands [4] Div. Medical Oncology, Dept. Internal Medicine, GROW, Maastricht University Medical Centre, Maastricht, The Netherlands [5] Leiden University Medical Center, Department of Gastroenterology and Hepatology, The Netherlands [6] Radboud University Medical Center, Department of Surgery, Nijmegen, the Netherlands [7] Tergooi Hospitals, Department of Internal Medicine, Hilversum, The Netherlands [8] Erasmus University Medical Center, Department of Medical Oncology, Rotterdam, The Netherlands [9] Cancer Center Amsterdam, Cancer Treatment and Quality of Life, Amsterdam, The Netherlands [10] Lava Therapeutics, Utrecht, The Netherlands
*Corresponding author

Abstract

Background: The prognosis of patients with advanced pancreatic ductal adenocarcinoma (PDAC) can be improved by FOLFIRINOX (5-fluorouracil, leucovorin, oxaliplatin and irinotecan). Upon progression after a therapy-free interval it is not uncommon to reintroduce FOLFIRINOX, depending on the response during and the progression free interval after FOLFIRINOX. The aim of this study is to provide an overview of the use and effectiveness of FOLFIRINOX reintroduction in daily practice.

Patients and Methods: Patients with locally advanced and metastatic PDAC diagnosed between 2015-2018 who started systemic treatment with palliative intent were selected from the Netherlands Cancer Registry (NCR). Overall and progression free survival (OS, PFS) were evaluated using Kaplan-Meier curves with log-rank tests.

Results: In this cohort (n=2092), most patients were treated with first line FOLFIRINOX (n=1381; 66%). Median OS from diagnosis until death was 9.0 months. A total of 388 patients (28%) received subsequent systemic therapy after first line FOLFIRNOX; 119 of 388 patients (30,7%) were re-treated with FOLFIRINOX after a minimum of 3 months treatment interruption while 269 patients were treated with other subsequent systemic therapy (majority gemcitabine plus nab-paclitaxel or gemcitabine monotherapy). Median therapy-free interval between first line FOLFIRINOX and FOLFIRINOX reintroduction was 7.0 months (p25-p75: 4,6-10,6). Patients underwent a median of 5 cycles (range: 1-32) for the initial treatment and 5 cycles (range: 1-28) for the reintroduction of FOLFIRINOX. Median OS after diagnosis of patients who received FOLFIRINOX reintroduction was 23.4 months. Median PFS after start of FOLFIRINOX reintroduction was 6.8 months.

Conclusion: Reintroduction of FOLFIRINOX after at least 3 months therapy-free interval is used in daily practice and seems a reasonable treatment option based on a favorable OS and PFS for in a small subset of patients.

Introduction

The prognosis of pancreatic ductal adenocarcinoma (PDAC) is poor (1, 2) and ranks among the leading causes of cancer mortality in the EU (3). Patients are frequently diagnosed in an advanced stage (4) in which palliative chemotherapy is the only treatment available. Compared to historic gemcitabine monotherapy (5), both FOLFIRINOX (oxaliplatin, irinotecan and fluorouracil in combination with leucovorin) and gemcitabine + nab-paclitaxel combination therapy have shown a significant overall survival (OS) improvement, both in clinical studies (6, 7) and according to real world data (8). ESMO and NCCN guidelines for pancreatic cancer (European Society for Medical Oncology (esmo.org); https://www.nccn.org) recommend FOLFIRINOX as the preferred first line treatment both in the locally advanced and metastatic setting for patients in a good clinical condition.

There is no consensus on the optimal duration of FOLFIRINOX in patients with a response or disease control, but the number of cycles is typically maximized to 12 (6 months) based on recommendations in the landmark study of Conroy et al. (6). After initial chemotherapy with FOLFIRINOX a chemotherapy holiday or maintenance treatment can be considered according to the NCCN Guidelines for PDAC (https://www.nccn.org). In daily practice, chemotherapy holidays are frequently used to preserve quality of life (QOL) after 1st line FOLFIRINOX (9). For patients who experienced clinical benefit (defined as stable disease or response) and manageable toxicity of first line FOLFIRINOX, re-introduction of FOLFIRINOX can be considered when disease progresses after a 3-6 months therapy-free interval. However, there is no clinical evidence that addresses this issue or help guide this clinical treatment decision.

Therefore the aim of this study was to present a comprehensive overview of the utilization, OS and progression free survival (PFS) of FOLFIRINOX reintroduction in routine daily practice.

Patients and methods

Patient selection

All adult patients newly diagnosed with PDAC between 2015 and 2018 who started systemic treatment were identified in the Netherlands Cancer Registry (NCR) for inclusion in this retrospective cohort study. The NCR is a population based database, hosted by the Netherlands Comprehensive Cancer Organization (IKNL) and covers all patients with a newly diagnosed malignancy in the total Dutch population of 17.2 million people in 2018. Notification sources were the Dutch nationwide pathology databank (PALGA) and supplemented with the Dutch National Hospital Care Registration (LBZ). In order to select patients who were treated with palliative intent, we excluded patients who underwent resection of the primary tumor or another local treatment with curative intent, e.g. neoadjuvant chemoradiotherapy in the PREOPANC trial (10), and patients with a documented curative intent treatment plan at diagnosis (based on arterial and venous involvement of the primary tumor and TNM tumor stage (https://dpcg.nl)). Patient characteristics (sex, age, performance status, previous cancer diagnosis, comorbidities), tumor (TNM stage, tumor histology, location of metastases), treatment (systemic treatment, radiotherapy, other local therapy) were recorded from the hospital's electronic health record system by trained registrars of the NCR. Response data were also recorded from the hospital's electronic health record system when available. Vital status was obtained by annual linkage to the Municipal Personal Records Database. This study was designed in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (11). The scientific committees of the Dutch Pancreatic Cancer Group (DPCG) and the Netherlands Cancer Registry (NCR) approved the study. Medical ethical approval was not required.

Palliative systemic therapy

In the selected patient group, type and timing of cancer treatments between diagnosis and death (or up to 3 years after diagnosis) were collected from the NCR database, as well as date(s) of progression. Systemic therapy regimens were classified as follows: FOLFIRINOX (5-FU in combination with irinotecan and oxaliplatin), gemcitabine + nab-paclitaxel, gemcitabine monotherapy, and other less commonly used regimens. Second line therapy was defined as treatment with agents not used in first line Reintroduction of FOLFIRINOX was defined as

a second treatment episode (minimum one cycle) with FOLFIRINOX after a minimum of 3 months (90 days) without any systemic treatment (therapy-free interval). Treatment duration intervals were calculated from start until stop date of the specific regimen. When patients continued fluorouracil-based therapy in a monotherapy or doublet regimen with oxaliplatin/irinotecan within 90 days after FOLFIRINOX, the stop date of the last agent in this regimen was used as the stop date of the FOLFIRINOX treatment. Retreatment with fluorouracil and oxaliplatin/irinotecan in a monotherapy or doublet regimen after a 3 months therapy-free interval was not classified as FOLFIRINOX reintroduction. Clinical benefit was defined as a documented radiologic complete or partial response (CR, PR) or stable disease (SD).

Statistical Analysis

Data in this study were analyzed using SPSS statistics 28 (IBM). OS was defined as the interval from diagnosis until death from any cause or otherwise noted, censored at last follow up date, updated on February 1, 2022. PFS was defined from start of reintroduction of FOLFIRINOX to documented progression. Data on progression were not available for all patients. In patients without documented progression of FOLFIRINOX reintroduction (n=36), time to progression was censored at time of last visit. However, several of these patients deceased within 30 days after the last visit or deceased (in the hospital) on the same day as the last hospital visit. Sensitivity analysis was performed with death within 30 days after the last hospital visit defined as a progression event. Median OS and PFS were analyzed using Kaplan Meier curves. A log rank test (Mantel-Cox) was used to compare OS differences in treatment groups. In addition, patients who deceased or stopped treatment within 30 days after FOLFIRINOX reintroduction were compared with other patients with reintroduction.

Results

First line systemic therapy

A total of 2092 patients who received systemic treatment with palliative intent for PDAC were included. Median age was 65 years (range 25-87). The majority of patients had distant metastases at time of diagnosis (74.7%) and the primary tumor was histologically or cytologically confirmed in 93% (table 1). Patients in this cohort

were treated with first line FOLFIRINOX (n=1381, 66%), gemcitabine monotherapy (n=360, 17.2%), gemcitabine + nab-paclitaxel (n=250, 12%) or other/unknown regimens (n= 101, 4.8%). Six percent of patients were treated with a local therapy in addition to chemotherapy (stereotactic radiotherapy (SRT), radiofrequency ablation (RFA) or irreversible electroporation (IRE)). The majority of patients had died at the time of analysis (n=2066, 98.8%). Median OS from diagnosis differed significantly between patients who were treated with first line FOLFIRINOX (9.0 months; 95% confidence interval (CI), 8.5 to 9.4), gemcitabine + nab-paclitaxel (7.0 months; 95% CI, 6.1 to 7.9) and gemcitabine monotherapy (4.8 months; 95%CI, 4.3 to 5.2) ($p<0.001$). First line treatment was administered for a median of 2.6 months (range 0-21.2; n=1373, missing in 7), 2.3 (range 0-21.5; n=249, missing in 1) and 1.4 months (range 0-18.4; n=357, missing in 3) for respectively FOLFIRINOX, gemcitabine + nab-paclitaxel and gemcitabine monotherapy.

Subsequent systemic therapy after first line FOLFIRINOX

Subsequent systemic therapy after first line FOLFIRINOX was started in 388 patients (28% of 1381). A total of 119 patients (31% of 388) received FOLFIRINOX reintroduction (> 3 months treatment interruption after first line FOLFIRINOX); 155 patients (40%) were treated with second line gemcitabine + nab-paclitaxel and 67 patients (17%) with gemcitabine monotherapy (table 1). Other second line treatments were started in 46 patients (12%).

FOLFIRINOX reintroduction

Incidence and treatment

The 119 patients who received FOLFIRINOX after > 3 months treatment interruption had a median age of 64 years and 57.1% presented with metastatic disease. The median therapy-free interval between the end of first line FOLFIRINOX and reintroduction was 7.0 months (range 3.1-35.7 months; p25-p75: 4.6-10.6 months). The number of FOLFIRINOX cycles for each treatment group is shown in table 2. Patients who were treated with FOLFIRINOX reintroduction received more often > 8 cycles of FOLFIRINOX in first line compared to all patients treated with FOLFIRINOX in first line (48 vs 27%). Patients received a median of 5 cycles during FOLFIRINOX reintroduction (range 1-28 cycles). While for each treatment episode the first treatment adjustment (if applicable) was documented, further information on dose reductions at the start of treatment, dose intensity, and toxicity of treatment were not documented. At least 64 of 119 patients (53.8%)

Table 1. Baseline patient characteristics in subsequent patient groups: all patients with palliative systemic treatment, first line FOLFIRINOX, FOLFIRINOX reintroduction after > 3 months therapy-free interval after first line FOLFIRINOX, second line nab-paclitaxel +gemcitabine after first line FOLFIRINOX and second line gemcitabine monotherapy after first line FOLFIRINOX. *Two patients treated with capecitabine-oxaliplatin-irinotecan were also considered as FOLFIRINOX. Abbreviations: SRT, stereotactic radiotherapy; RFA, radiofrequent ablation; ***IRE***, Irreversible electroporation.

Baseline characteristics	palliative systemic treatment (total)	palliative FOLFIRINOX as first systemic treatment*	FOLFIRINOX reintroduction	Second line Nab-paclitaxel +gemcitabine	Second line Gemcitabine Monotherapy
N	2092	1381	119	155	67
Median age (y, range)	65 (25-87)	63 (25-83)	64 (39-79)	61 (25-80)	63 (43-82)
Age > 70y (%)	563 (26,9)	229 (16,6)	25 (21)	22 (14,2)	12 (17,9)
Male (%)	1138 (54,2)	760 (54,9)	58 (48,7)	88 (56,8)	34 (50,7)
distant metastasis (%)	1563 (74,7)	1014 (73,2)	68 (57,1)	99 (63,9)	53 (79,1)
WHO PS (%)					
0-1	1357 (64,9)	983 (71,2)	96 (80,7)	118 (76,2)	51 (76,1)
2	210 (10,0)	90 (6,5)	8 (6,7)	9 (5,8)	2 (3)
3-4	32 (1,5)	14 (10,1)	-	2 (1,2)	1 (1,5)
Missing	493 (23,6)	294 (21,3)	15 (12,6)	26 (16,8)	13 (19,4)
No co-morbidity (%)	1057 (50,5)	778 (56,3)	66 (55,5)	85 (54,8)	37 (55,2)
Local treatment (%)	125 (6,0)	112 (8,1)	36 (30,3)	17 (11)	2 (3)
SRT	71 (3,4)	65 (4,7)	14 (11,8)	9(5,8)	1 (1,5)
RFA	26 (1,2)	22(1,6)	8 (6,7)	6 (3,9)	1 1,5)
IRE	28 (1,3)	25 (1,8)	14 (11,8)	2 (1,3)	

needed a dose reduction after FOLFIRINOX reintroduction, compared to 73 of 119 patients (61.3%) during first line FOLFIRINOX. In this cohort 36 patients (30.3%) were treated with local therapy (majority LAPC; n=34, 94.4%).

Table 2. FOLFIRINOX cycles during first line and reintroduction.

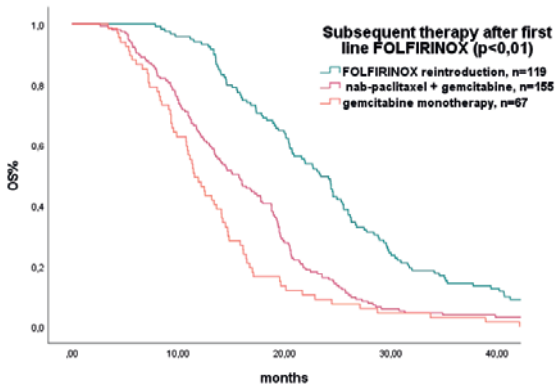
FOLFIRINOX cycles	First line palliative FOLFIRINOX (all patients)	First line palliative FOLFIRINOX (FOLFIRINOX reintroduction group)	First line palliative FOLFIRINOX (Second line Nab-paclitaxel +gemcitabine group)	First line palliative FOLFIRINOX (Second line gemcitabine monotherapy group)	FOLFIRINOX reintroduction
N	1381	119	155	67	119
Median FOLFIRINOX cycles	5 (range 1-32)	8 (range 2-19)	8 (range 1-31)	7 (range 1-20)	5 (range 1-28)
patients with > 8 cycles	363 (26,9%)	56 (48,3%)	66 (43,4%)	24 (35,8%)	23 (20%)
patients with > 1 cycle	1124 (83,3%)	116 (100%)	144 (94,7%)	61 (91%)	102 (88,7%)
missing (n)	31	3	3	-	4

OS, PFS and response

The median OS from diagnosis until death was 23.4 months (95%CI, 21.0 to 25.8), 15.7 months (95%CI, 13.4 to 18.0) and 11.5 months (95%CI, 10.0 to 13.1) for patients who were treated with FOLFIRINOX reintroduction, gemcitabine + nab-paclitaxel and gemcitabine monotherapy in second line respectively fig 1a, p<0.01). Patients who presented with or without metastatic disease had a similar median OS from diagnosis until death after FOLFIRINOX reintroduction (22.7 months (95%CI, 19 to 26.4) versus 24.4 months (95%CI, 21.7 to 27.1)(p=1.0)). The median OS from start of FOLFIRINOX reintroduction until death was 8.3 months (95%CI, 6.8 to 9.7)(fig 1b) while the median OS from stop of FOLFIRINOX reintroduction until death was 4.4 months (95% CI, 3.2 to 5.6). Median PFS after start of FOLFIRINOX reintroduction (n=119) was 6.8 months (95% CI, 6.1 to 7.5) (fig 1c). In sensitivity analysis, with death within 30 days after the stop date of last FOLFIRINOX treatment as a progression event, a shorter PFS of 5.4 months (95% CI, 3.7-7.0) was found. All patients (response unknown in 12/119) who received FOLFIRINOX reintroduction experienced clinical benefit during first line FOLFIRINOX as best response compared to 51,6% and 53,7% of patients who received second line gemcitabine + nab-paclitaxel and gemcitabine monotherapy (response unknown in 33/155 and 14/67 patients). More than half of the patients (n=70, 58.8%) experienced clinical benefit as best response during FOLFIRINOX reintroduction (table 3, response unknown in 20%). To identify patients without benefit of FOLFIRINOX reintroduction we analyzed which patients died or stopped shortly after start of FOLFIRINOX reintroduction. Twenty-four of 119 patients (20.2%) deceased (n=4) or stopped FOLFIRINOX (n=20) within 30 days after start of FOLFIRINOX reintroduction. The median therapy-free interval from first line FOLFIRINOX until first FOLFIRINOX reintroduction was 5.4 months (range 3.1 -35.7 months; < 6 months in n=13/24, 54.2%) compared to 7.1 months (range 3.1 -31.7 months; < 6 months in n=32/95, 33.7%) for patients who did or did not die or stop within 30 days after FOLFIRINOX reintroduction. A small group of patients (n=15, 53% metastatic disease) was even treated with a second reintroduction of FOLFIRINOX without any other systemic therapy in between and had a median OS of 31 months from diagnosis and 17.4 months from start of first reintroduction.

Table 3. Best response of FOLFIRINOX treatment for patients treated with FOLFIRINOX reintroduction; after first line and reintroduction of FOLFIRINOX (CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease).

Best response	n	n
	First line FOLFIRINOX (%)	FOLFIRINOX reintroduction (%)
CR	4 (3)	1 (1)
PR	62 (52)	23 (19)
SD	41 (35)	46 (39)
PD	0	25 (21)
unknown	12 (10)	24 (20)
total	119	119



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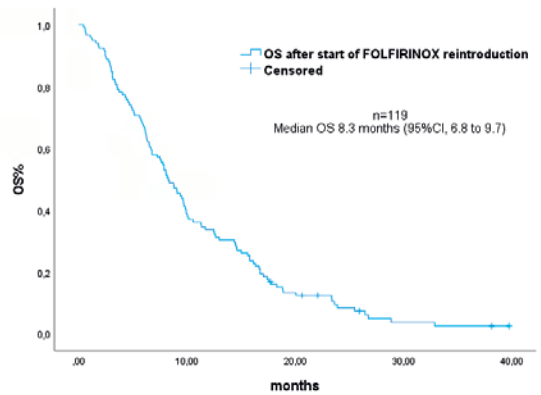

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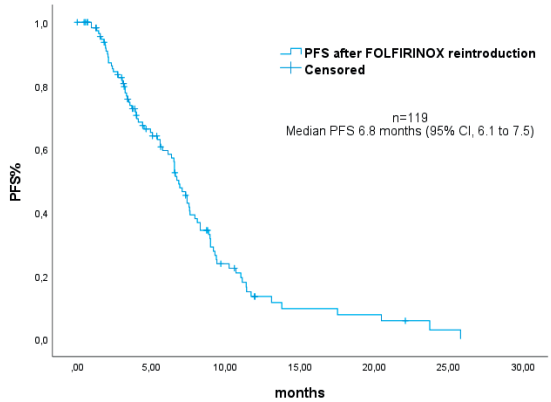

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Figure 1. a. OS (from diagnosis) by subsequent treatment group; FOLFIRINOX reintroduction, nab-paclitaxel + gemcitabine and gemcitabine monotherapy.
b. OS after start of FOLFIRINOX reintroduction.
c. PFS after start of FOLFIRINOX reintroduction.

Discussion

This retrospective cohort study focused on FOLFIRINOX reintroduction after a minimum of 3 months therapy-free interval following first line palliative FOLFIRINOX for advanced PDAC. Patients who were treated with FOLFIRINOX reintroduction (119 of 1381 patients who received first line FOLFIRINOX) had a favorable median OS of 23 months from diagnosis and 8.3 months from reintroduction of FOLFIRINOX with a median therapy-free interval of 7 months between first line and FOLFIRINOX reintroduction. Combination chemotherapy until adverse events or disease progression necessitate a therapeutic change is the established standard treatment for PDAC (12). To our knowledge this is the first study that focuses on reintroduction of FOLFIRINOX after a treatment interruption following first line palliative FOLFIRINOX, although a maintenance approach after induction chemotherapy is explored in several studies. Support was found in the PANOPTIMOX-PRODIGE trial for maintenance fluorouracil-leucovorin after 4 months induction FOLFIRINOX in metastatic PDAC although this strategy did not reduce chemotherapy-induced neurotoxicity (13). Another maintenance strategy for metastatic disease was investigated in the randomized SEQUENCE phase I/II trial, exploring standard gemcitabine/nab-paclitaxel versus alternating maintenance gemcitabine-nab-paclitaxel and modified FOLFOX, resulting in improved OS (13.2 v 9.7 months; HR, 0.68; p = .02) for the alternating

approach (ASCO 2022) (14). In a retrospective study, maintenance fluorouracil monotherapy after FOLFIRINOX was associated with an OS of 18.3 months without OS or PFS difference between fluorouracil or FOLFIRI maintenance (15). The OS of patients retreated with FOLFIRINOX without maintenance therapy in our cohort does not seem detrimental compared to the survival rates in the maintenance studies, although these data cannot be directly compared. To specify maintenance treatment to tumor biology, olaparib maintenance can be considered for patients with a germline BRCA1/2 mutation, but unfortunately without a significant OS benefit (16). Patients (n=3) who received olaparib in our study were not included in the FOLFIRINOX reintroduction cohort. The optimal approach after first line FOLFIRINOX is not known. A second line cohort study reported an OS of 7.5 months after start of second line gemcitabine + nab-paclitaxel after 5FU based combinations (17). An OS of 11.5 and 12.4 months from diagnosis was reported for gemcitabine based second line therapy after FOLFIRINOX failure (18). In a previous Dutch NCR cohort the median OS was 11.2 months for patients who were treated with all types of systemic second line therapy (8). A comparison with our data is not possible due to the fact that second line studies focused on patient with FOLFIRINOX resistant disease. Our study has several limitations. First, due to the design of a non-randomized retrospective cohort study, patient and treatment selection bias influences a comparison between different treatments after first line FOLFIRINOX. In the landmark study of Conroy et al. the median number of FOLFIRINOX cycles administered was 10 (range 1 - 47) compared to 5 cycles (range 1 - 32) of first line FOLFIRINOX in our cohort (6). A substantial number of patients in our cohort (n=226, 16,7%) was only treated with 1 cycle of first line FOLFIRINOX, suggesting not all patients in our cohort would have been eligible for the landmark study. Patients who are eligible for FOLFIRINOX reintroduction are a selection of patients with clinical benefit on first line FOLFIRINOX, limited residual toxicity and a sufficient clinical condition. For example, patients in our cohort who could be treated with FOLFIRINOX reintroduction or other subsequent therapies were treated with more cycles of FOLFIRINOX in first line compared to all first-line FOLFIRINOX patients. In addition, patients treated with FOLFIRINOX reintroduction less frequently presented with metastatic disease at diagnosis (57 vs. 73% all first line FOLFIRINOX patients). One can envision that this difference translates into more patients being in a sufficient condition to be retreated with FOLFIRINOX. Second, information on WHO performance status and treatment response was missing in many patients

in this real world cohort without structured reporting as in randomized clinical trials. Third, the exact moment of disease progression (according to RECIST) was not structurally reported in the medical charts. This may have influenced PFS intervals, as detection of progressive disease may have been omitted in case of clinical deterioration. After sensitivity analysis, with death shortly after FOLFIRINOX termination redefined as a progression event, PFS was reduced by more than a month. Fourth, only the type of the first therapy adjustment (e.g. delay or dose reduction) after start of systemic treatment was registered for each treatment episode. No data were available about dose reductions at the start of treatment, dose intensity and toxicity separately which makes it difficult to draw conclusions about safety. At last, we cannot rule out an effect on OS of a local treatment. Patients who were treated with a FOLFIRINOX reintroduction more often were treated with an additional local therapy compared to patients who were treated with second line gemcitabine plus nab-paclitaxel and gemcitabine monotherapy (30 vs 11 and 3%). We found a favorable OS but also a large range of survival and treatment cycles after FOLFIRINOX reintroduction in our nationwide database. For example, one fifth of patients stopped FOLFIRINOX or died soon after start of FOLFIRINOX reintroduction, while others were treated for many months. In the patients with poor results after FOLFIRINOX reintroduction, the therapy-free interval after first line FOLFIRINOX was shorter (5.4 vs. 7.1 months) compared to patients who were treated > 30 days with FOLFIRINOX reintroduction. The findings of this study can be used as background information (in absence of RCTs for second line treatments after FOLFIRINOX) when reintroduction of FOLFIRINOX is considered in patients experiencing clinical benefit during first line FOLFIRINOX.

Conclusion

The present study provides a nationwide overview about the use of reintroduction of FOLFIRINOX in routine daily practice after at least a 3 months therapy-free interval in patients with advanced PDAC. Reintroduction of FOLFIRINOX can be a reasonable approach for those who are in a sufficient clinical condition and experienced clinical benefit, preferable leading to the possibility of a longer therapy-free interval, and limited toxicity during first line FOLFIRINOX.

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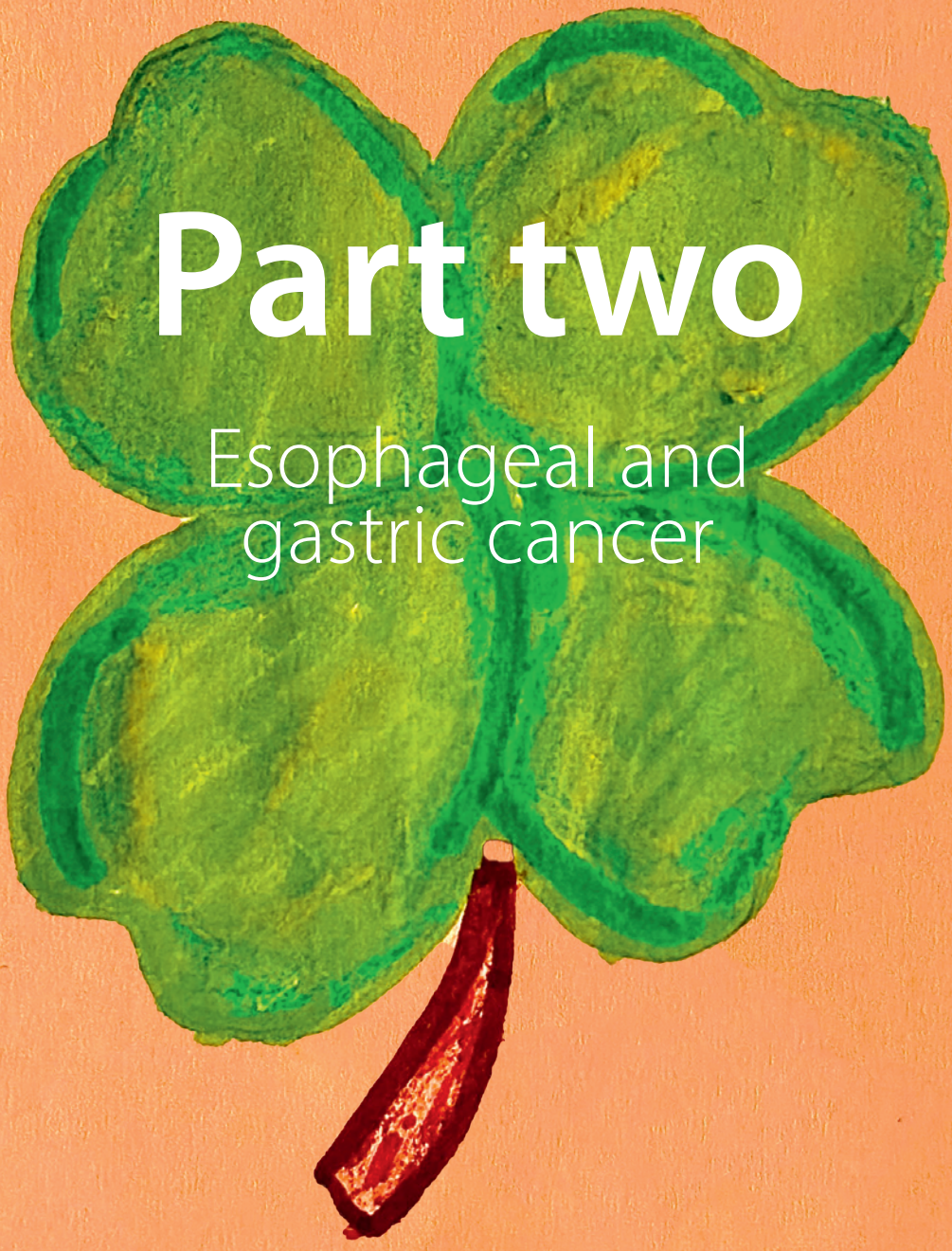

Part two

Esophageal and gastric cancer

Randomized phase 2 study of gemcitabine and cisplatin with or without vitamin supplementation in patients with advanced esophagogastric cancer

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A. A. van Zweeden[1,2], C. J. van Groeningen[2], R. J. Honeywell[1], E. Giovannetti[1], R. Ruijter[1], C. H. Smorenburg[3], G. Giaccone[4], H. M. W. Verheul[1], G. J. Peters[1], and Hans J. van der Vliet[1]*

[1]Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands [2]Department of Internal Medicine, Amstelland Hospital, Amstelveen, The Netherlands [3]Department of Internal Medicine, Noordwest Ziekenhuisgroep Alkmaar, Alkmaar, The Netherlands [4]Department of Medical Oncology, Georgetown University Medical Center, Washington, DC USA
*Corresponding author

Abstract

Purpose: Preclinical research and prior clinical observations demonstrated reduced toxicity and suggested enhanced efficacy of cisplatin due to folic acid and vitamin B12 suppletion. In this randomized phase 2 trial, we evaluated the addition of folic acid and vitamin B12 to first-line palliative cisplatin and gemcitabine in patients with advanced esophagogastric cancer (AEGC).

Methods: Patients with AEGC were randomized to gemcitabine 1250 mg/m2 (i.v. days 1, 8) and cisplatin 80 mg/m2 (i.v. day 1) q 3 weeks with or without folic acid (450 µg/day p.o.) and vitamin B12 (1000 µg i.m. q 9 weeks). The primary endpoint was response rate (RR). Secondary endpoints included overall survival (OS), time to progression (TTP), toxicity, and exploratory biomarker analyses. Cisplatin sensitivity and intracellular platinum levels were determined in adenocarcinoma cell lines cultured under high and low folate conditions *in vitro*.

Results: Adenocarcinoma cells cultured in medium with high folate levels were more sensitive to cisplatin and this was associated with increased intracellular platinum levels. In the randomized phase 2 clinical trial, which ran from October 2004 to September 2013, treatment was initiated in 78 of 82 randomized pts, 39 in each study arm. The RR was similar; 42.1% for supplemented patients vs. 32.4% for unsupplemented patients; p=0.4. Median OS and TTP were 10.0 and 5.9 months for supplemented vs 7.7 and 5.4 months for unsupplemented patients (OS, p=0.9; TTP, p=0.9). Plasma homocysteine was lower in the supplemented group (n = 20, 6.9 ± 1.6 (mean ± standard error of mean, SEM) µM; vs. 12.5 ± 4.0 µM; $p < 0.001$). There was no significant difference in the Cmax of gemcitabine and cisplatin in the two treatment groups.

Conclusion: Folic acid and vitamin B12 supplementation do not improve the RR, PFS or OS of cisplatin and gemcitabine in patients with AEGC.

Introduction

Esophagogastric cancer is one of the most common malignancies of the gastrointestinal tract worldwide. These cancers encompass malignant epithelial neoplasms located in all regions of the esophagus and stomach irrespective of the histological type. In the majority of cases, the malignancies are adenocarcinomas (AC) or squamous cell carcinomas (SCC). The incidence of SCC has largely remained constant over time, while the incidence of AC has increased[1]. Treatment for metastatic disease is palliative and frequently consists of combination chemotherapy and/or radiotherapy. The goals of palliative systemic chemotherapy are survival benefit and palliation of symptoms[2,3]. Cisplatin has been considered a key substance in combination regimens for metastatic gastro-esophageal cancer[4]. Results from a phase 2 study at our institute showed a response rate (RR) of 41% using the combination of cisplatin and gemcitabine, with manageable toxicity[5]. Treatment with pemetrexed plus cisplatin and vitamin supplementation resulted in superior survival time, time to progression, and response rates compared with treatment with cisplatin alone in patients with malignant mesothelioma in the EMPHACIS trial[6]. The majority of patients in this study received folic acid and vitamin B12. Vitamin suppletion significantly reduced toxicity of the chemotherapy and did not decrease efficacy parameters. Vitamin suppletion was found to be predictive of increased overall survival in a multivariate regression analysis of prognostic factors derived from this trial[7]. Preclinical evidence demonstrated that differences in the folate environment resulted in a different sensitivity of human cancer cell lines to cisplatin[8,9]. Tumor cells that are relatively cisplatin resistant require lower intracellular folate concentrations for growth[10]. In line, low tumor cell expression levels of the folate receptor (FR), which is a major influx transporter for folates in normal tissues and certain tumors[11], are associated with cisplatin resistance[12,13]. In this paper we first investigated the cisplatin sensitivity of adenocarcinoma cell lines grown under high or low folate conditions. Adenocarcinoma cells grown under high folate conditions were more sensitive to cisplatin and this was associated with higher intracellular platinum accumulation, providing a rationale for supplementation of patients with folates. Based on these *in vitro* data and the clinical suggestion of increased efficacy in mesothelioma patients we hypothesized that folate supplementation to patients would increase the sensitivity to cisplatin based treatment. We designed a randomized phase 2 trial in order to determine whether supplementation of folic

acid and vitamin B12 could increase the efficacy of gemcitabine and cisplatin in advanced esophagogastric cancer.

Patients and methods

Effect of folic acid supplementation on intratumoral accumulation of cisplatin and tumor cell sensitivity to cisplatin

In order to determine whether and how folic acid supplementation would affect sensitivity to cisplatin we tested the cisplatin sensitivity of two pairs of adenocarcinoma cell lines WiDr and CaCo2 and their sublines (WiDr/LF, CaCo2/LF/LV and CaCo2/LF/FA) adapted to grow under low folate conditions [13,14]. Due to the unavailability of modified esophageal adenocarcinoma cell lines, adenocarcinoma cell lines of colorectal origin were used for this purpose. Standard mycoplasma testing was performed. Wild type WiDr and CaCo2 are cultured in standard DMEM medium containing 8 μM folic acid, WiDr/LF and CaCo2/LF/LV have been selected to grow in folate-free RPMI medium supplemented with 2.5 and 1 nM leucovorin, respectively, while CaCo2/LF/FA is adapted to grow in RPMI medium supplemented with 1 nM folic acid. Sensitivity of these cells to cisplatin was determined by a 72 hr exposure to cisplatin alone or in combination with gemcitabine using the sulforodamide B (SRB) assay[15]. We also determined whether folate supplementation would affect the accumulation of cisplatin into these cells. Intracellular platinum concentrations were determined as described earlier[16].

Clinical study design and study population

The clinical study was a multicenter randomized open label phase 2 study comparing therapy with gemcitabine and cisplatin with or without vitamin B12 and folic acid supplementation. From October 2004 to August 2013, 82 patients were included in the study. The study recruited patients in the VU University medical center (VUmc) in Amsterdam, The Netherlands and the Noordwest Ziekenhuisgroep in Alkmaar, The Netherlands. Main inclusion criteria included histologically or cytologically confirmed metastatic or locally advanced unresectable advanced esophagogastric carcinoma (AEGC), squamous cell or adenocarcinoma, not amenable to curative treatment, measurable disease according to RECIST[17], age of at least 18 years, ECOG performance score of 0-2,

life expectancy of at least 12 weeks, adequate bone marrow function, adequate renal function, and adequate hepatic function. Prior surgery, chemotherapy and/or radiotherapy in the neo-adjuvant or adjuvant setting was allowed as long as the chemotherapy was completed at least 6 months prior to entry of the study. Written informed consent was obtained from all patients prior to inclusion into the study. Patients with known symptomatic metastasis in the central nervous system (CNS) or suffering from any serious concomitant systemic disorders incompatible with study treatment were not eligible. Other exclusion criteria were treatment with any investigational agent in the month prior to inclusion or prior diagnosis of other malignant disease (excluding adequately treated in situ carcinoma of the cervix and non-melanoma skin cancer, low grade prostate carcinoma or any other non-relapsed malignancy that was treated more than five years before diagnosis). Randomization was performed by the datamanagement center of the Integraal Kanker Center Amsterdam (IKA) using a computerized randomization system. The institutional Medical Ethical board of the VUmc and Noordwest Ziekenhuisgroep approved the trial, which was in accordance with the Declaration of Helsinki and Good Clinical Practice.

Study treatment

Patients were randomized to receive treatment with gemcitabine 1250 mg/m^2 intravenously (i.v.) on days 1 and 8 in combination with cisplatin 80 mg/m^2 i.v. on day 1 in a 3 weekly cycle with or without vitamin supplementation, further described as supplemented vs unsupplemented patients, respectively.

Vitamin supplementation consisted of folic acid 450 µg/24 h per os (p.o., starting at least one week prior to chemotherapy and finishing at least 3 weeks after the last treatment dose, and vitamin B12 1000 µg (1 vial intramuscularly, i.m.) every 9 weeks, starting 1 week before chemotherapy and finishing at least 3 weeks after the last treatment dose. Patients were treated with up to 6 cycles of chemotherapy. Study treatment was discontinued in case of progressive disease, unacceptable toxicity or upon patient request.

Endpoints

The primary endpoint of this study was to determine whether supplementation of folic acid and vitamin B12 could increase the response rate (RR) of patients with

advanced esophagogastric cancer treated with the combination of gemcitabine and cisplatin. Secondary endpoints were assessment of time to progression (TTP), defined as the time from randomization to progression and overall survival (OS), defined as the time from randomization to death. Further secondary objectives included the assessment of plasma homocysteine concentrations as an indication for plasma folic acid homeostasis. Moreover we investigated the effect of folate supplementation on plasma pharmacokinetics of cisplatin (total and free unbound), gemcitabine, the gemcitabin degradation product 2',2'-difluoro-2'-deoxyuridine (dFdU) and, in white blood cells (WBC), its active metabolite gemcitabine-triphosphate (dFdCTP). We also determined polymorphisms in the genes encoding methylenetetrahydrofolate reductase (MTHFR), which may affect folate homeostasis[11], and cytidine deaminase (CDA), that catalyzes the deamination of gemcitabine to dFdU[18].

Toxicity evaluation, dose adjustments and response assessment

Treatment toxicity was rated according to CTC version 2.0 (CTCAE v2.0 Cancer Therapy Evaluation Program, Common Terminology Criteria for Adverse Events, Version 2.0, DCTD, NCI, NIH, DHHS (http://ctep.cancer.gov). All serious adverse events (SAE) were collected from registration until 30 days after the last protocol treatment administration. Criteria for chemotherapy administration on day 1 of each cycle was delayed one week in case of neutropenia (absolute neutrophil count (ANC) of < 1.5 x 109/L) or thrombopenia (platelets < 100 x 109/L), renal toxicity (creatinine > 120 µmol/L, and/or creatinine clearance < 60 ml/min) or any non-haematological toxicity above CTC grade 1 or baseline. Gemcitabine was reduced on day 8 with 25% or 50% in case of grade 2 or 3 neutropenia or grade 1 or 2 thrombopenia, respectively. Platelets < 50 x 109/L or neutrophils < 0.5 x 109/L were reason to omit gemcitabine on day 8. The dose of gemcitabine was reduced 50% in case of grade 3 non-haematological toxicity (except emesis) and discontinued in case of grade 4 AE's. The doses of gemcitabine and cisplatin were reduced with 25% after a two week treatment delay due to toxicity, neutropenic fever, grade 4 neutropenia and/or thrombopenia lasting over one week or thrombopenia associated with bleeding. Cisplatin was reduced or discontinued in case of grade ≥2 peripheral neuropathy or grade ≥2 renal toxicity or other grade ≥3 non-haematological toxicity (except emesis). No dose escalations were allowed. Unacceptable toxicity was defined as failure to recover from side effects after a treatment delay of a maximum of 3 weeks, requirement of a third dose

reduction, the repeated occurrence of grade 3 or 4 non-haematological toxicity or drug-induced pneumonitis [3] grade 2 or according to investigator's judgement. Tumor assessments by CT scan of chest and abdomen were performed every 6 weeks until disease progression according to RECIST[17]. A baseline scan was done within 4 weeks before initiation of study therapy. Disease status, date of progression, date of death and subsequent lines of therapy were collected during regular follow-up visits. TTP was defined as the time from randomization to progression. OS was defined as the time from randomization to death.

Assessment of potential predictive parameters

Plasma pharmacokinetics of gemcitabine, its metabolite dFdU and cisplatin were measured during treatment in the first 20 patients in order to assess whether vitamin supplementation would affect either gemcitabine or cisplatin pharmacokinetics. We also determined the concentration of dFdCTP in WBC and the homocysteine concentration in these 20 patients. The other patients were monitored for homocysteine before randomization and at the beginning of each 3rd chemotherapy cycle (one week after vitamin B12 administration). In order to assess these parameters, blood was collected in heparinized tubes containing tetrahydrouridine to prevent conversion of gemcitabine to dFdU. After centrifugation the plasma was taken off and stored at -20° C until analysis. The intermediate layer between plasma and red blood cells containing the WBC was layered on Ficoll-Hypaque, centrifuged and the buffy coat with the WBC was washed, counted and the pellet was frozen in liquid nitrogen until analysis for dFdCTP. Gemcitabine, dFdU and dFdCTP were measured with validated HPLC assays[16]. Homocysteine was measured as described earlier[19]. In order to determine the amount of total and free (non-protein) bound platinum species, one part was immediately frozen at -20° C until analysis (total platinum), and the other part was mixed with ethanol, incubated overnight at -20° C, and centrifuged. The supernatant contained free platinum[20]. Free plasma platinum and total plasma platinum (free and protein-bound platinum) were determined using flameless atomic absorption spectroscopy[20,16].

The 79A>C (rs2072671) CDA and 667C>T MTHFR polymorphism were analyzed in respectively 37 and 20 patients in this study to asses a possible association with response, survival and toxicity.

Statistical analysis

Based on a hypothesized 1.5 fold improvement in the RR from an anticipated 33% in the non-supplemented arm to 50% in the vitamin supplemented arm, a sample size of 82 patients (41 per study arm) was required. If the true RR difference between the study regimens would be ^{3}15%, there would be an approximate 90% probability of selecting the true superior arm. The unpaired t-test was used to compare RR and the stratified log rank test was used to compare survival rates between treatment groups. An intention to treat analysis was used for TTP and OS. OS and TTP were calculated using Kaplan-Meier estimates. The correlation between homocysteine levels in both treatment groups was determined with a 2-tailed t-test. P values < 0.05 were considered statistically significant.

Results

High folate status increases sensitivity to cisplatin

Under normal (high folate) cell culture conditions CaCo2 cells were more sensitive to cisplatin than WiDr cells (IC_{50} 1.2 ± 0.2 μM for CaCo2 vs. 6.4 ± 0.5 μM for WiDr, means ± SEM, $p<0.001$). However, when cultured under low folate (LF) conditions these cell lines were 2-5 fold less sensitive to cisplatin (IC_{50} for CaCo2-LF sublines CaCo2-LF/LV, 5.6 ± 0.5 μM; $p<0.01$ and CaCo2-LF/FA 3.4 ± 0.3 μM; $p<0.02$), and for WiDr/LF 10.1 ± 0.1 μM; $p<0.02$) as shown in figure 1. Addition of gemcitabine to cisplatin resulted in a slight increase in cisplatin sensitivity, but the difference in IC_{50} between high and low folate containing medium remained the same.

In order to investigate the mechanism behind the observed difference in cisplatin sensitivity of tumor cells cultured in medium containing different folate concentrations, tumor cells were exposed to 20 μM cisplatin for 24 hr. WiDr cells accumulated more platinum than WiDr-LF cells (50 ± 0.5 vs 28 ± 3 pmol/10^6 cells, respectively; $p<0.0001$), while CaCo2 cells (96 ± 16 pmol/10^6 cells) accumulated more platinum than CaCo2-LF/LV (58 ± 8 pmol/10^6 cells; $p<0.05$) or CaCo2-LF/FA (44 ± 6 pmol/10^6 cells; $p<0.02$). Co-incubation with gemcitabine resulted in a slight increase in cisplatin accumulation, especially under LF conditions (not shown). Overall, these data demonstrate that high folate conditions can increase sensitivity of adenocarcinoma cells to cisplatin, and that this is associated with higher intratumoral platinum accumulation, providing a rationale for supplementation of patients with folates.

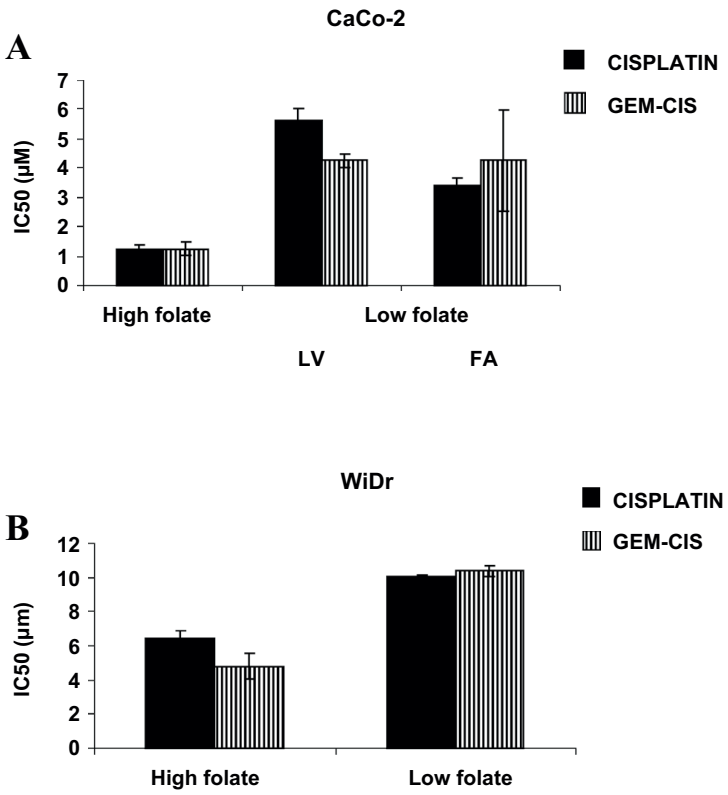


Figure 1. High folate conditions can increase sensitivity of adenocarcinoma cells to cisplatin. CaC02 and WiDr adenocarcinoma cell lines were treated with cisplatin and gemcitabine under high folate or low folate conditions. (A) CaC02 and sublines CaCo2/LF/LV and CaCo2/LF/FA; (B) WiDr. CaCo2-LF sublines CaCo2-LF/LV (IC50 5.6 ± 0.5 µM; p<0.01), CaCo2-LF/FA (IC50 3.4 ± 0.3 µM; p<0.02), and WiDr/LF (IC50 10.1 ± 0.1 µM; p<0.02) under low folate (LF) conditions were 2-5 fold less sensitive to cisplatin compared to culture under high folate conditions. Abbreviations: LF, low folate; FA, folic acid reduced into the nM range; LV, leucovorin reduced into the nM range.

Patient characteristics

A total of 82 patients were randomly assigned to each treatment arm (41 patients in each arm). Baseline characteristics were generally well matched between the two treatment arms (Table 1). The mean age of patients was 61 years (range 35-83). The majority of patients were male, and most (72%) suffered from advanced esophageal cancer. In 85-90% of patients the ECOG performance score was 0-1. Less than 10% of the supplemented patients and none of the unsupplemented patients had undergone prior treatment for esophagogastric carcinoma (two patients received palliative radiotherapy of the primary tumor, one patient received

neoadjuvant chemotherapy and one patient received prior chemoradiation). Treatment was initiated in 78 patients. Four patients did not receive chemotherapy after randomization. One patient, allocated to the vitamin group, deceased unexpectedly before the first chemotherapy while another patient in the same treatment group was not eligible due to neutropenia. Two patients allocated to the treatment arm without vitamin suppletion were not eligible due to increasing renal impairment. These four patients could not be monitored for response but were included in the intention to treat analysis for TTP and OS.

Table 1. Baseline patient and disease characteristics.

	Supplemented patients N=41	**Unsupplemented patients N=41**
Characteristic		
Mean age (range)	61 yr (50-78)	61 (35-82)
Gender (female/male)	8 / 33	8 / 33
Primary tumor (stomach /esophagus)	11 /30	12 / 29
Tumortype (SCC/AC)	8/33	8/33
Performance status		
PS 0	14 (34%)	12 (29%)
PS 1	23 (56%)	23 (56%)
PS 2	2 (5%)	4 (10%)
PS unknown	2 (5%)	2 (5%)
Prior therapy	4 (10%)	0

Safety and tolerability

The overall incidence of grade 3-5 AEs was comparable between the two treatment groups and probably caused by the chemotherapy (Table 2). Grade 3 leukopenia was the most common severe toxicity in supplemented patients (22%), while fatigue was the most common severe toxicity (24%) in unsupplemented patients. Three supplemented patients suffered from grade 4 thrombopenia. Grade 4 thrombopenia was reported in one unsupplemented patient. Two supplemented patients were diagnosed with an ischemic cerebrovascular accident (CVA) after two treatment cycles (grade 4 neurologic toxicity) and one patient was diagnosed with a hemorrhagic CVA three days after day 1 of the first chemotherapy cycle (grade 4 hemorrhage) and received no further study treatment. Two unsupplemented patients deceased shortly after the first chemotherapy cycle, in 1 case probably

due to cardiac arrhythmias likely caused by cardiac metastases and in the other case due to the occurrence of cardiac failure. These events were considered to be most likely related to cisplatin chemotherapy and underlying predisposing conditions of the patients. These three patients could not be monitored for response but were included in the intention to treat analysis for PFS and OS.

Twenty patients (in both treatment groups) were treated with darbepoetin alfa for chemotherapy induced anemia [21] with a hemoglobin response in 15 of these 20 patients. Darbepoetin did not increase toxicity.

Table 2. Treatment related grade 3-5 AEs per study arm. Percentages are rounded to whole numbers. For each grade 3/4/5 adverse event the maximum toxicity was noted per patient.

Adverse event	Supplemented patients (n=41), n (%)			Nonsupplemented patients (n=41), n (%)		
	Grade			Grade		
	3	4	5	3	4	5
Febrile neutropenia	2 (5)			1 (2)		
Leukopenia	9 (22)			4 (10)		
Trombopenia	4 (10)	3 (7)		4 (10)	1 (2)	
Anemia	6(15)			2 (5)		
Fatigue	4 (10)			10 (24)		
Cardiac	1 (2)			1 (2)		2 (5)
Neurologic	1 (2)	2 (5)		5 (12)		
Ototoxicity				1 (2)		
Pulmonary				1 (2)		
Nausea	4 (10)			3 (7)		
Vomiting	2 (5)			2 (5)		
Anorexia	2 (5)			5 (12)		
Liver	1 (2)					
Diarrhea				1 (2)		
Pain				1 (2)		
Skin	1 (2)					
Renal/bladder	5 (12)					
Hemorrhage	1 (2)	1 (2)		2 (5)		
Infection	1 (2)			1 (2)		

Response rate, overall survival and time to progression

Response rate

Thirty-eight supplemented patients and 37 unsupplemented patients were evaluable for response. The RR was 42.1% (n=16) for supplemented patients, all partial responses (PR). The RR for unsupplemented patients was 32.4% (n=12), and consisted of PR in 29.7% (n=11) and a complete response (CR) in 2.7% (n=1) of patients. The RR was not significantly different between the two treatment groups, p=0.4.

Overall survival and time to progression

The median OS in this study was 10.0 months (range 0.3-42.6) for the supplemented arm vs 7.7 months (range 0.03-46.7) for the unsupplemented arm. This difference was not statistically significant (p=0.9). One patient was lost to follow up and was censored for the OS analysis. According to the prespecified intention to treat analysis all other randomized patients were included in the survival analysis, including one patient who deceased between randomization and the start of study treatment and one patient who was treated with epirubicin and oxaliplatin instead of cisplatin-gemcitabine due to renal impairment. This patient was not included in the TTP analysis. Vitamin supplementation did not lead to a significantly different median TTP, 5.9 months (range 1.4-33.5) for supplemented patients vs 5.4 months (range 1.4-30.9) for unsupplemented patients (p=0.9). The Kaplan-Meier curves for OS and TTP are shown in figure 2.

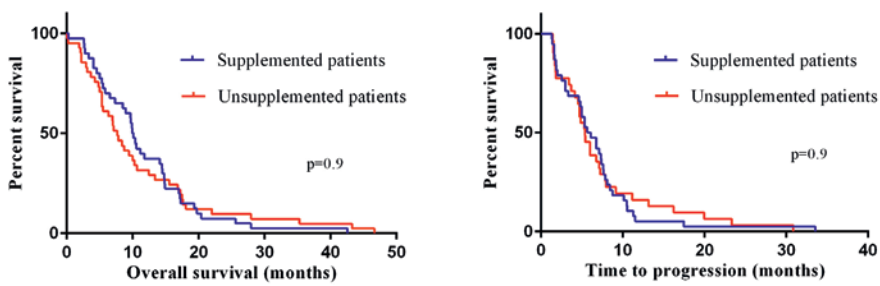
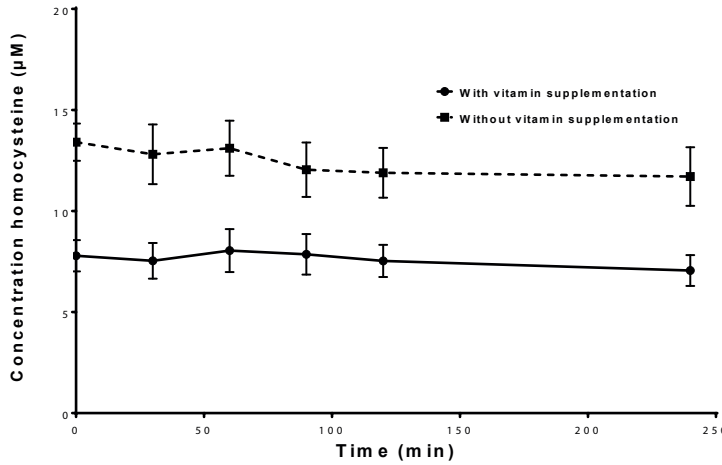


Figure 2. Kaplan-Meier curve for OS and TTP. The dotted line represents the supplemented arm while the black line represents the unsupplemented arm. OS and TTP were not significantly different between the supplemented vs. the unsupplemented patients (median OS 10.0 months; range 0.3-42.6 vs. 7.7 months; range 0.03-46.7; p=0.9; median TTP 5.9 months; range 1.4-33.5 vs. 5.4 months; range 1.4-30.9; p=0.9).

Pharmacokinetic monitoring and assessment of potential prognostic parameters.

Following vitamin supplementation, homocysteine levels were lower in supplemented patients vs unsupplemented patients (mean 6.9 ± 1.6 μM; range 6.2±7.2 vs. 12.5 ± 4.0 μM; range 11.7±13.4; $p < 0.001$) as shown in figure 3. This difference was expected since homocysteine levels are inversely related to folate and vitamin B12 consumption. Compared to pre-randomization levels, vitamin supplementation decreased homocysteine levels in supplemented patients while homocysteine increased when patients were randomized to the unsupplemented arm. This illustrates compliance to the allocated treatment arm.The maximum concentration (C_{max}) of gemcitabine was identical for patients in both treatment groups (n = 20; C_{max} 53.4 ± 18.6 (mean ± SEM) μM for supplemented vs. 53.2 ± 15.0 μM for unsupplemented pts). However, vitamin supplementation did change gemcitabine pharmacokinetics. For example, supplementation resulted in increased levels of the gemcitabine metabolite dFdU (Fig. 3; $p<0.05$), and in addition also resulted in an increase in the formation of the active metabolite of gemcitabine dFdCTP (peak levels at 30 min 255 ± 190 vs 133 ± 93 pmol/10^6 cells and at 90 min 298 ± 245 vs 226 ± 101 pmol/10^6 cells; $p>0.1$), though these results were not statistically significant. Vitamin supplementation led to a small, but not statistically significant, increase in total cisplatin levels (total platinum 15.7 ± 2.7 μM in the vitamin group vs 14.8 ± 1.4 μM for patients without vitamin supplementation), and a significant increase in free (non-protein bound) platinum levels: 4.7 ± 1.7 μM in the vitamin group vs. 3.6 ± 0.7 μM without vitamin supplementation ($p<0.05$). Genetic polymorphisms in the folate metabolizing enzyme MTHFR 677C>T were measured in 20 patients while polymorphisms in the gemcitabine metabolizing enzyme CDA 79A>C were measured in 37 patients. For the CDA gene, neither the OS (p=0.56; Log-rank; Mantel-Cox test), TTP (p=0.61; Log-rank; Mantel-Cox test) nor the RR (p=0.46, ANOVA test) differed significantly between the patients with the AA, CC or AC variant, although analyzed patient numbers may be too small to formally rule out smaller differences (table 3). Similarly, the incidence of grade 3 toxicity of any cause was not statistically significantly different between patients with either of the three polymorphisms (p=0.9).The OS (p=0.90 Log-rank; Mantel-Cox test), TTP (p=0.89 Log-rank; Mantel-Cox test) and RR (p= 0.42 ANOVA test) were not significantly different for patients with a TT, CC or CT MTHFR 677. Again numbers may be considered too small for a reliable comparison. Grade 3 toxicity of any cause was equally distributed between the different polymorphisms.

A



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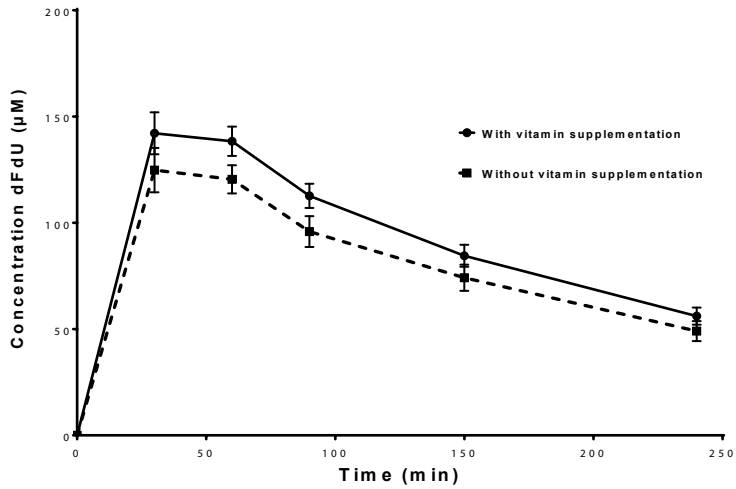

Figure 3. Plasma concentrations of homocysteine and dFdU in vitamin supplemented and unsupplemented the patients from the pharmacokinetics cohort. (A) The black line represents the supplemented arm while the dotted line represents the unsupplemented arm. Values are means ± SEM from 10 patients in each cohort. Homocysteine levels were lower in supplemented patients vs unsupplemented patients (mean 6.9 ± 1.6 µM; range 6.2±7.2 vs. 12.5 ± 4.0 µM; range 11.7±13.4; $p < 0.001$); (B) The black line represents the supplemented arm while the dotted line represents the unsupplemented arm. Supplementation resulted in increased levels of the gemcitabine metabolite dFdU ($p<0.05$). Values are means ± SEM from 10 and 7 patients, respectively.

Table 3. CDA and MTHFR gene polymorphisms in relation to outcome and toxicity. Polymorphisms in the gene for CDA were measured in 37 patients. The AA variant was found in 22 patients, the CC variant in 7 patients and AC variant in 8 patients. Polymorphisms in the gene for MTHFR were measured in 20 patients. The TT variant was found in 2 patients, the CC variant in 6 patients and CT variant in 12 patients. Abbreviations: OS, overall survival (median months); TTP, time to progression (median months); RR, response rate (%); PR, partial response; CR, complete response; SD, standard deviation.

	AA **n=22**	CC **n=7**	AC **n=8**	TT **n=2**	CC **n=6**	CT **n=12**
Clinical parameter						
OS (months)	7.8 (SD 9.1)	17.3 (SD 13.7)	10.1 (SD 3.1)	11.0 (SD 12.4)	5.0 (SD 19.3)	9.8 (SD 11.0)
TTP (months)	5.5 (SD 12.9)	9.0 (SD 8.6)	6.8 (SD 2.0)	10.5 (-)	1.9 (SD 12.8)	6.4 (SD 9.0)
PR/CR	n=8	n=3	n=5	n=1	n=1	n=6
RR (%)	36	43	63	50	17	50
Grade 3 toxicity	n=12 (55%)	n=4 (57%)	n=5 (63%)	n=1 (50%)	n=3 (50%)	n=6 (50%)
Grade 4 toxicity	-	-	N=1 (13%)	-	-	-

Discussion

In this study we demonstrate that the combination of gemcitabine and cisplatin can be considered an effective palliative chemotherapeutic regime in patients with AEGC. The median combined OS of 9.2 months and TTP of 5.4 months in our study is comparable with the currently commonly used first line palliative chemotherapy regimens for AEGC. The current standard first line palliative chemotherapy for AEGC consists of triplet chemotherapy regimens such as EOX (epirubicin, oxaliplatin and capecitabine, OS/PFS 11.2/7.0 months), ECX (epirubicin, cisplatin, capecitabine, OS/PFS 9.9/6.7 months) or EOF (epirubicin, oxaliplatin and fluorouracil, OS/PFS 9.3/6.5 months)[22] or doublet therapies (fluorouracil, leucovorin in combination with oxaliplatin or cisplatin, OS/PFS resp. 10.7/5.8 vs. 8.8/3.9 months)[23], or capecitabine and oxaliplatin, OS 8 months[24]. A recent meta-analysis showed a limited survival benefit of triplet chemotherapy with an increased risk of toxicity when compared to doublet chemotherapy[25]. Cisplatin and gemcitabine have a different side effect profile compared with oxaliplatin based chemotherapy regimens. Cisplatin is associated with a higher incidence of grade 3 to 4 neutropenia, alopecia, thromboembolism, and renal dysfunction while peripheral neuropathy and diarrhea is a more frequent side effect of oxaliplatin [26,27]. The intravenous administration route of cisplatin and

gemcitabine can be a relevant consideration for patients with AEGC and problems with the passage of food (and oral medication such as e.g. capecitabine) as a result of obstruction caused by the primary tumor. Therefore, the here employed cisplatin and gemcitabine treatment combination can be considered a reasonable or perhaps even preferred palliative treatment option for AEGC patients with e.g. signs of dysphagia or preexistent neuropathy.

This multicenter randomized phase 2 trial was designed to investigate whether supplementation of folic acid and vitamin B12 resulted in an improved clinical outcome in AEGC patients treated with the combination of cisplatin and gemcitabine chemotherapy. Addition of folic acid and vitamin B12 to this chemotherapy backbone did not significantly increase the RR which was 42.1% (n=16) in the vitamin group vs 32.4% (n=12) in the chemotherapy alone group. The difference in RR between the study arms did not meet the prespecified target RR of 50% nor a 15% difference in RR between the two treatment groups. The median OS was not significantly different with or without vitamin suppletion (10.0 months vs 7.7 months). The median TTP was similar in both treatment groups (5.9 months for supplemented patients vs 5.4 months for unsupplemented patients). Baseline characteristics of the patients in both study arms were well balanced. Vitamin supplementation did not result in an apparent decrease in the incidence of grade 3-5 adverse events.

As homocysteine levels are inversely related to folate and vitamin B12 consumption, the measured lower concentration of homocysteine in patients receiving concomitant vitamin supplementation is indicative of the biological activity of the employed vitamin supplementation and is in support of adequate patient compliance[28]. Though the use of second line chemotherapy or experimental therapy was not specifically documented in this trial, its use could potentially affect differences in OS between the two study groups. The impact of second line chemotherapy in AEGC was very limited if at all present and is unlikely to substantially confound our data.

Our study results contrast with the previously reported beneficial effects observed when folic acid and vitamin B12 were added to the combination of cisplatin and pemetrexed and cisplatin monotherapy[6]. Apart from the fact that a different patient group was studied, both studies also differed in their design as our study

was randomized for the addition of vitamins while the study of Vogelzang et al. was not randomized for vitamin suppletion. The discrepancy could also be related to specific effects of folate and vitamin B12 on the efficacy of pemetrexed that do not occur with the cisplatin and gemcitabine combination used in this study[29]. Indeed, in the phase 3 study of Vogelzang et al. the benefit of adding vitamin suppletion was predominantly observed in the group of patients treated with pemetrexed and cisplatin. Moreover, as in our study cisplatin was combined with gemcitabine a potential positive effect of folate suppletion on cisplatin sensitivity (e.g. an increased exposure to free platinum) might have been masked by effects on gemcitabine metabolism as e.g. degradation of gemcitabine to dFdU was increased in supplemented patients. In earlier studies an association between increased gemcitabine deamination and a lower response rate and survival were reported[30]. The formation of the active metabolite of gemcitabine dFdCTP in white blood cells, included as a surrogate biomarker for tissue accumulation, was initially increased. The levels of dFdCTP and the effect of cisplatin are in line with other studies of gemcitabine-cisplatin combination therapy[31]. However, this difference did not persist and may therefore preclude a clinically relevant increase of dFdCTP levels in tissues, which is necessary for an optimal effect of gemcitabine[32]. One can also not exclude that the potentiating effects of vitamin supplementation, as found in patients with mesothelioma, differ between tumor types. The 79A>C polymorphism in the CDA gene and the 667C>T polymorphism in the MTHFR gene were measured in a subgroup of patients. We found no correlation with RR, OS, TTP or severe toxicity although numbers were small and the study was not powered for this analysis.

The results of this trial are important for daily practice since vitamin supplement use is very common among patients with cancer[33,34] Reasons for vitamin suppletion include an expected reduced toxicity of chemotherapy, an expected enhanced efficacy of cancer treatment in combination with vitamin use and an expected improvement in general well-being. Complementary medicine is very often not evidence based[35], but in this randomized trial no efficacy benefit was found of vitamin suppletion. In recent years, clinical trials for advanced esophagogastric cancer have focused more on triple-drug regimens. These consist of chemotherapy with tumor-specific targeted therapies, e.g. therapies targeting Her2, c-Met or VEGFR[36-38]. These approaches are likely to be further

developed and expanded in an effort to improve the still dismal perspectives of patients suffering from metastatic esophagogastric cancer.

In conclusion this phase 2 trial has demonstrated that folic acid and vitamin B12 supplementation does not improve the RR, PFS or OS of cisplatin and gemcitabine in patients with AEGC. We here show that the combination of gemcitabine/cisplatin is a reasonable alternative treatment schedule for patients with AEGC in case of dysphagia or preexistent neuropathy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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The prognostic impact of circulating miRNAs in patients with advanced esophagogastric cancer during palliative chemotherapy

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Annette A. van Zweeden[1,2], Roza C.M. Opperman[1], Richard J. Honeywell[1], Godefridus J. Peters[1,3], Henk M.W. Verheul[1,4], Hans J. van der Vliet[1,5], Dennis Poel[1,4*]

[1]Amsterdam UMC, Vrije Universiteit Amsterdam, Cancer Center Amsterdam, Department of Medical Oncology, Amsterdam, Netherlands,[2] Amstelland Hospital, Internal Medicine, Amstelveen, Netherlands, [3] Department of Biochemistry, Medical University of Gdansk, Gdansk, Poland, [4] Department of Medical Oncology, Radboud University Medical Center, Nijmegen, Netherlands, 5 Lava Therapeutics, Yalelaan 60, Utrecht, Netherlands
*Corresponding author

Abstract

The prognosis of patients with advanced oesophageal cancer (EC) and gastric cancer (GC) is poor. Circulating microRNAs (ci-miRNAs) may have prognostic and predictive value to improve patient selection for palliative treatment. The purpose of this study is to assess the prognostic and predictive value of specific ci-miRNAs in plasma of patients with EC and GC treated with first-line palliative gemcitabine and cisplatin. Droplet digital PCR (ddPCR) was used to quantify miR-200c-3p, miR-375, miR-21-5p, miR-148a-3p, miR-146a-5p, miR-141-3p and miR-218-5p in plasma from 68 patients. ci-miRNA expression was analyzed in relation to overall survival (OS), progression-free survival (PFS), and response to chemotherapy. ci-miRNA levels were detectable in 36 baseline (71%) samples and in 14 (47%) follow-up samples. Increased circulating miR-200c-3p in GC showed a trend ($p=0.06$) towards a shorter OS. High circulating miR-375 was associated with a longer OS ($p=0.02$) in patients with esophageal adenocarcinoma (EAC). No significant difference was observed in ci-miRNA expression between paired pre- and on-treatment samples. ci-miRNA expression was not associated with response to chemotherapy. ci-miRNAs can be measured in plasma samples of patients treated with first-line palliative chemotherapy using ddPCR despite prolonged storage in heparin. Elevated circulating miR-375 might be a prognostic marker for patients with EAC.

Introduction

Esophagogastric cancer is one of the most common malignancies of the gastrointestinal tract worldwide. The most common histologic subtypes of this disease are adenocarcinomas (AC) and squamous cell carcinomas (SCC) [1]. The incidence of oesophageal adenocarcinoma (EAC) (predominantly in the distal oesophageal and esophagogastric junction) has increased in Western countries and accounts for > 60 percent of all oesophageal cancers (EC) in the United States [2]. Treatment for metastatic disease frequently consists of palliative chemotherapy and/or radiotherapy[3]. The median overall survival (OS) after palliative chemotherapy is around 11 months [4,5]. Though, response and survival rates vary among patients. Prognostic and predictive biomarkers have been proven beneficial for treatment selection. For example, a survival benefit has been observed for anti-HER2 directed therapies in patients with HER2-neu overexpressing gastro-oesophageal junction tumours [6].

MicroRNAs (miRNAs) are a class of small (approximately 22 nucleotides in length) non-coding RNAs that mediate gene expression through complementary binding to the 3'untranslated regions (3'UTRs) of target messenger RNA (mRNA) genes [7]. miRNAs that target tumour suppressor genes function as oncogenes while miRNAs that target oncogenes exert a tumour suppressor function[8] in different cancer types[9]. Tumour or plasma specific miRNA signatures of patients with EC and gastric cancer (GC) were previously associated with clinical outcome [10-27]. The definite role of miRNAs in EC and GC is unclear due to conflicting study outcomes, [13,15,16] and limited data are available about the function of miRNAs in plasma of patients treated with palliative chemotherapy. In order to investigate the prognostic and predictive value of ci-miRNAs in patients treated with palliative gemcitabine-cisplatin based chemotherapy for advanced EC and GC, the expression levels of miR-200c-3p, miR-375, miR-21-5p, miR-148a-3p, miR-146a-5p, miR-141-3p and miR-218-5p, which were selected based on literature, were quantified with droplet digital PCR (ddPCR) in plasma samples collected during a randomized phase II clinical trial.

Materials and Methods

Study population and blood samples

Between October 2004 and September 2013, a total of 82 patients with histologically confirmed locally advanced or metastatic EC or GC were enrolled in a randomized phase II study that was performed in the Amsterdam UMC, location VUmc, and the Noordwest Ziekenhuisgroep in Alkmaar, both in the Netherlands. The institutional Medical Ethical board of the VUmc and Noordwest Ziekenhuisgroep approved the trial, which was in accordance with the Declaration of Helsinki and Good Clinical Practice. Written informed consent was obtained from all patients prior to inclusion into the study. The primary endpoint of this study was to determine whether supplementation of folic acid and vitamin B12 could increase the response rate (RR) of patients with advanced esophagogastric cancer treated with the combination of gemcitabine and cisplatin. The addition of folic acid and vitamin B12 did not improve RR, PFS or OS in this study [28]. Secondary endpoints included survival and pharmacokinetic analysis. Seventy-eight patients were randomized to treatment with up to six cycles of cisplatin and gemcitabine in a three-weekly cycle with or without vitamin B12 and folic acid suppletion. Blood samples were drawn prior to onset of the study and collected in BECTON DICKINSON Vacutainer Heparin Tubes in which 10 µg/ml tetrahydrouridine (THU) was spiked in to avoid conversion of gemcitabine into 2',2'-difluorodeoxyuridine (dFdU). This was done for pharmacokinetic analyses as previously reported. Follow-up samples were drawn before the third (n=35) and the sixth (n=12) cycle of chemotherapy. After centrifugation the plasma was taken off and stored at -25 °C until analysis. The plasma samples collected during the course of this study were used for ci-miRNA analysis.

miRNA selection

Seven miRNAs were selected for measurement based on the literature (Table 1). PubMed search criteria included micro RNA and gastric or oesophageal cancer (MESH, all fields, and similar articles). miRNAs were selected if evidence was presented in at least two independent publications. miR-375, miR-200c-3p, miR-21-5p and miR-148a-3p were selected for measurement in patients with EAC and ESCC. miR-200c-3p, miR-141-3p, miR-146a-5p and miR-218-5p were selected for measurement in patients with GC. These miRNAs were all previously detected in blood samples [29].

Table 1. Literature background for miRNA selection in oesophageal and gastric cancer. Abbreviations: H, high expression; L, low expression; ESCC, oesophageal squamous cell cancer; EAC, oesophageal adenocarcinoma; GC, gastric cancer; C, circulating; T, tissue; HR, hazard ratio; p, p value, all p values lower than 0.05 were noted as <0,05; *, HR missing; ↓, shorter OS; ↑, longer OS; ** for this study relation with PFS was reported, no relation with OS.

miRNA	No. studies	HR for OS	(95% CI)	Relation miRNA-OS	p-value	No. pts, histology	Specimen	Ref
miR-21 (H)	5	1.87	1.4-2.6	↓	< 0.05	504 ESCC/ EAC	T C	[25]
	1	*		↓	< 0.05	38 ESCC	C	[17]
miR-375 (H)	6	0.55	0.4-0.7	↑	< 0.05 < 0.05	723 ESCC	T C	[21]
	9	1.69	1.5–2.0	↓	0.05	1230 ESCC	T C	[21] [30]
miR-148a (H/L) miR-148a (L)	1	*		↓(H:EAC) ↓(L: SCC)	< 0.05	45 ESCC/ EAC	T	[10]
	1	*		↓	< 0.05	49 ESCC	T	[27]
miR-141(L)	1	*		↓	< 0.05	95 GC	T	[18]
	1	*		↓	< 0.05	30 GC	T	[31]
miR-200c-3p (H)	1	*		↑	< 0.05	51 GC	T/C	[16]
	1	2.24	1.1-4.6	↓	< 0.05	52 GC	C	[13]
	1	4.01	2.8-10.0	↓	< 0.05	98 GC	C	[15]
	1	1.67	1.1-2.4	↓	< 0.05	157 ESCC	C	[30]
	1	*		↓**	< 0.05	64 ESCC	C	[24]
miR-146a(L)	3	2.60	1.63-4.13	↓	<0.05	213 GC	T	[12]
	1	1.53	1.06-2.26	↓	<0.05	90 GC	T	[20]
miR-218 (L)	1	3.16	1.06-9.40	↓	<0.05	68 GC	C	[19]
	1	3.19	1.55-8.37	↓	<0.05	112 GC	T	[32]

RNA isolation, heparinase treatment, RT-qPCR, sample quality and droplet digital PCR

RNA isolation of heparinized plasma samples from the included patients was performed using the miRNeasy Serum/ Plasma Advanced Kit (QIAGEN, Venlo, the Netherlands) according to the manufacturer`s protocol, with adjustments as described previously [33] and stored at -80°C until further analysis. Heparin contamination interferes with the reverse transcriptase polymerase chain reaction [34]. Therefore the RNA samples were treated with Bacteroides heparinase I (NEB, Leiden, the Netherlands, 12.000 U/ml) [35]. In order to optimize the heparinase treatment of plasma RNA, RNA from plasma samples of 3 healthy donors was isolated and treated with heparinase under different conditions. Different volumes of isolated plasma RNA (10, 20 or 30 µl) were incubates with different quantities of heparinase I (6, 12 or 18U). Synthetic cel-miR-39-3p (spiked during the RNA isolation procedure) was quantified with RT-qPCR as read-out for heparinase treatment efficiency in these samples. As a positive control for this experiment

plasma RNA was isolated from the same healthy donors drawn in a 6 ml EDTA tube (BECTON DICKINSON). Constant factors in all the heparinase reactions were heparinase buffer (5 µl) (NEB), Protector RNAse inhibitor (2000 units, 1.25 µl) (ROCHE, Woerden, the Netherlands) supplemented with nuclease-free H_2O to a total reaction volume of 50 µl. All reaction conditions were incubated for two hours at 30°C. cDNA synthesis was performed with the QIAGEN miRCURY LNA kit according to the supplied protocol, with an adjustment for RNA input. Two µl RNA and five µl H_2O were replaced with seven µl RNA treated with heparinase. The cDNA was diluted 1:40 and cel-miR-39-3p was quantified using miRCURY LNA miRNA PCR Assays (QIAGEN)[33]. RNA isolated from the H630 colorectal cancer cell line spiked with cel-miR-39-3p was used as a positive control and nuclease-free water served as a non-template negative control in each experiment. The expression of cel-miR-39-3p detected after RNA isolation is correlated to the efficiency of the RNA isolation, and therefore used as a control measure for RNA quality. All samples with a cel-miR-39-3p Cq of 33 or higher were excluded for further analysis. To test the stability of synthetic cel-miR-39-3p during heparinase treatment conditions, cel-miR-39-3p was quantified using RT-qPCR after incubation for two hours at 30°C in nuclease-free H_2O.

miRNAs are expressed in red blood cells (RBC), lymphoid or myeloid blood cells. RBC-expressed miRNAs were reported to be increased by 20- to 30-fold in haemolysed plasma. Non–RBC-associated miRNAs were not increased in haemolysed samples[36]. miR-375 and miR-141-3p are non–RBC-associated miRNAs. miR-21-5p, miR-146a-5p, miR-148a-3p and miR-200c-3p are present in RBC. Therefore, the hemolytic index for each sample was defined as described previously [33]. Samples with a hemolytic index > 10 were excluded since adverse effects on non–RBC-associated miRNAs cannot be excluded [37].

The following miRCURY LNA PCR assays were used for miRNA quantification using ddPCR: cel-miR-39-3p (diluted 1:1), miR-21-5p, miR-375, miR-200c-3p, miR-148a-3p, miR-146a-5p, miR-141-3p and miR-218-5p (QIAGEN). A thermal gradient for each primer assay was performed using colon cancer cell lines (HT29, ATCC and H630) and head and neck squamous cell carcinoma cell line VU-SCC-120. The annealing temperatures for the thermal gradient PCR ranged between 52°C and 62°C. All ddPCR procedures and reaction volumes were performed according to the manufacturer's protocol (QX200 Droplet Digital PCR System from BIO-RAD,

BIO-RAD Laboratories, Veenendaal, the Netherlands). The data were analyzed with Quantasoft software (BIO-RAD). The cut-off for the number of quantified droplets was set on at least 10.000. Expression is defined as miRNA copy per 1 µl input normalized to cel-miR-39-3p [38]. Continuous miRNA expression levels measured with ddPCR were converted to a dichotomous variable using the median expression as a threshold. All experiments were carried out in accordance with institutional guidelines and regulations.

Statistical analysis

Overall survival and progression-free survival differences between patients divided based on median ci-miRNA expression in the baseline samples were calculated by performing a Kaplan-Meier analysis and a log-rank test. Analysis between the paired baseline and follow-up samples was performed using the Wilcoxon matched-pairs-signed rank test. ci-miRNA expression was compared between patients with or without clinical benefit (CB) of the chemotherapy for the subgroups GC, EC and EAC or GC (miR-200c-3p) using the Mann-Whitney U test. Patients who did not start with chemotherapy were excluded from this analysis. The Mann-Whitney U test was used to compare the ci-miRNA expression in the samples with vitamin supplementation. The statistical analysis was performed using GRAPHPAD PRISM 8 Software.

Results

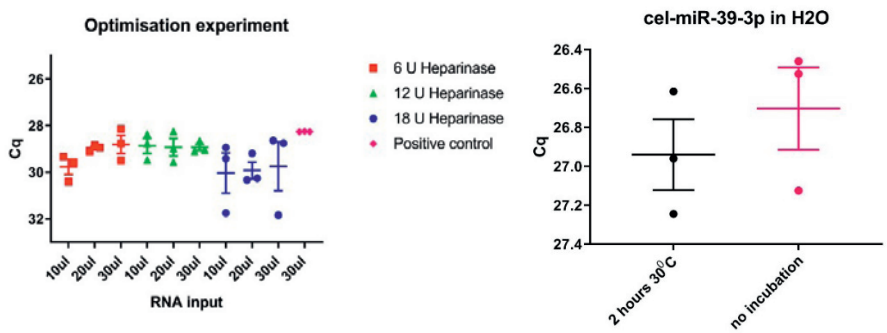
Patient samples

A total of 225 plasma samples were collected originating from 68 patients during the randomized phase two study. From 63 patients a baseline sample was available (14GC; 49EC). A total of 53 follow up samples were available of these 63 patients (12GC; 41EC).

Heparinase treatment optimization and sample inclusion

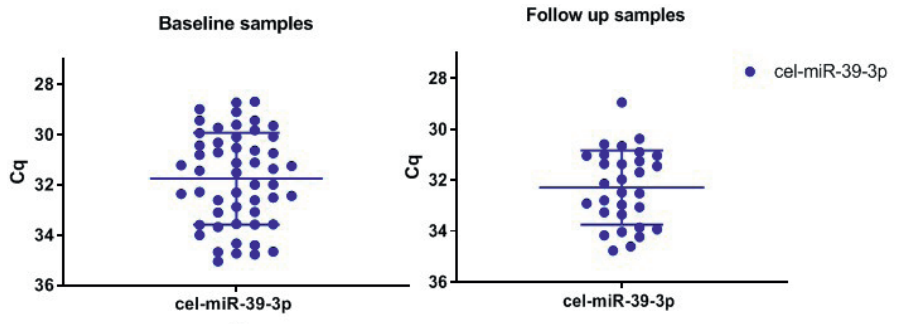
miRNA detection in the heparinized patient samples by RT-PCR was not possible without heparinase treatment. To optimize the heparinase treatment different RNA volumes incubated with different heparinase quantities were tested. Cel-miR-39-3p quantification in RNA isolated from EDTA plasma without heparinase was used as a positive control. The mean Cq value of cel-miR-39-3p in this positive

control group was 28.26 ± 0.01 SEM (pink diamonds, Figure 1). Twenty µl RNA incubated with 6 U heparinase (28.96 ± 0.08 SEM) and 30 µl RNA incubated with 12 U heparinase (28.93 ± 0.14 SEM) were the most efficient volumes for cel-miR-39-3p detection (Figure 1). Thirty µl RNA incubated with 12 U heparinase was selected for this study. To define if cel-miR-39-3p is degraded during the heparinase treatment, it was incubated for 2 hours at 30oC in H_2O. No significant effect was observed when a similar concentration of cel-miR-39-3p as spiked-in during the RNA isolation was incubated for 2 hours at 30^0C in H_2O (Figure 1). Cel-miR-39-3p was measurable in duplicate in 52 (82,5%) baseline samples and in 30 (56,6%) follow-up samples. Twenty-seven samples were excluded (15 (28,8%) baseline and 12 (40%) follow-up) due to a low (Cq ≤33) cel-miR-39-3p expression (Figure 2). Five samples were excluded (1 (1,9%) baseline and 4 (22,2%) follow-up) based on a high (>10) hemolytic index. As a result, 36 (69,2%) of the 52 baseline samples (10GC;19EAC;8ESCC) and 14 (46,7%) of the 30 follow-up samples (7GC;5EAC;2ESCC) were included for further analysis (Supplementary Figure S1). Baseline characteristics of the patients with measurable plasma samples that complied with the quality criteria are described in Table 2.



(a) (b)

Figure 1. (a) The effect of different heparinase quantities (Units, U) in combination with different RNA volumes on the detection of cel-miR-39-3p by RT-qPCR. Each point in the graph represents the raw Cq value of duplicate RT-qPCR measurements of cel-miR-39-3p in plasma RNA isolated from three different healthy donors. RNA input is shown on the x-axis and raw Cq value of cel-miR-39-3p is shown on the y-axis; (b) The detection of 20 fM cel-miR-39-3p in water by RT-qPCR after different incubation times. The H2O samples were not treated with heparinase. Each point in the graph represents the raw Cq value on the Y-axis of duplicate RT-qPCR measurements from two different samples on the x-axis.



(a) (b)

Figure 2. (a) Cel-miR-39-3p expression levels in all measurable baseline samples (n=52); (b) Cel-miR-39-3p expression levels in all measurable follow-up samples (n=30). Cel-miR-39-3p is shown on the x-axis and the Cq value is shown on the y-axis.

Table 2. Patient characteristics with measurable plasma samples. Abbreviations: yrs, years; GC, gastric cancer; EAC, oesophageal adenocarcinoma; ESCC, oesophageal squamous cell carcinoma.

Characteristics	GC N = 10	EAC N = 18	ESCC N = 8
Age (yrs), median (range)	63.1 (50-75)	61 (35-82)	61.8 (52-71)
Gender			
Female	3	3	3
Male	7	15	5
Prior surgery /therapy			
No			
Yes	9	16	7
	1	2	1

Thermal gradient droplet digital PCR

A thermal gradient ddPCR was performed to determine the optimal annealing temperature for each primer assay. For all primer assays 52.7°C was the most optimal annealing temperature (Supplementary Figure S2)

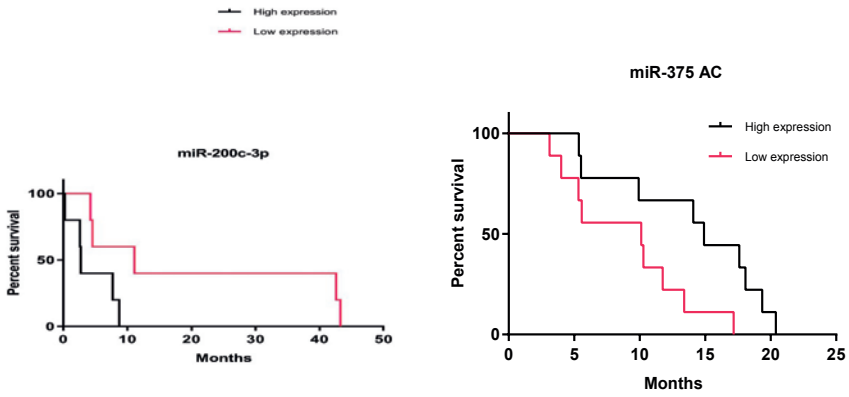
Association of ci-miRNA expression with overall survival and progression-free survival

GC

High baseline expression levels of miR-200c-3p showed a trend towards a worse OS (2.8 vs. 11.1 months; HR=2.9 95% CI:0.7-11.8, p=0.06). No significant relation was found between miR-141-3p, miR-146a-5p and miR-218-5p expression and OS or progression-free survival (PFS) in patients with GC (Table 3 and Figure 3a).

Table 3. Association of miRNA expression with OS and PFS. Abbreviations: n, number of patients; H - L, high vs low miRNA expression; HR, hazard ratio; OS, overall survival (months); PFS, progression-free survival (months); P, p-value; vs., versus; GC, gastric cancer; EAC, oesophageal adenocarcinoma; ESCC, oesophageal squamous cell carcinoma

Origin	miRNA	n; H-L	HR (95% CI)	*P*	Median OS (CI)	HR (95% CI)	*P*	Median PFS (CI)
GC	**141-3p**	4-3	1.6 (0.4-7.3)	0.5	5.1 (0.3-8.7) vs. 4.6 (2.7-43.3)	1.4 (0.3-6.0)	0.7	6.8 (0.3-8.7) vs. 4.5 (1.8-33.5)
GC	**200c-3p**	5-5	2.9 (0.7-11.8)	0.06	2.8 (0.3-8.7) vs. 11.1 (4.3-43.3)	2.4 (0.6-9.5)	0.1	1.6(0.3-8.7) vs. 8.1(1.8-33.5)
GC	**146a-5p**	5-5	1.4 (0.4-4.9)	0.6	7.7 (2.8-11.1) vs. 4.6 (0.3-43.3)	1.5 (0.4-5.2)	0.5	6.8 (1.6-8.7) vs. 4.6 (0.3-33.5)
GC	**218-5p**	3-3	1.7 (0.3-8.8)	0.5	2.8 (0.11-8.7) vs. 4.6 (0.3-43.3)	1.7 (0.3-8.8)	0.5	1.6 (0.11-8.7) vs. 4.5 (0.3-23.3)
EAC	**375**	9-9	0.4 (0.1-1.1)	0.02	14.9 (5.4-20.4) vs. 10.1 (3.1-17.2)	0.5 (0.2-1.3)	0.1	6.1 (3.0-17.5) vs. 5.4 (1.5-7.7)
EAC	**200c-3p**	9-9	0.7 (0.3-1.8)	0.4	10.3 (3.1-0.4) vs. 11.8 (4.0-18.1)	1.2 (0.5-3.0)	0.7	5.4(1.5-17.5) vs. 6.0 (1.5-11.3)
EAC	**21-5p**	9-9	0.7 (0.3-1.9)	0.5	10.3 (3.1-20.4) vs. 11.8 (4.0-18.1)	1.0 (0.4-2.6)	1.0	5.5 (1.5-17.5) vs. 5.9 (1.5-11.3)
EAC	**148a-3p**	8-9	0.5 (0.2-1.4)	0.2	14.2 (5.4-20.4) vs. 10.3 (3.1-18.1)	0.9 (0.3-2.2)	0.7	5.8 (3.0-17.5) vs. 5.4 (1.5-11.3)
GC + AEC	**200c-3p**	14-14	1.5 (0.7-3.1)	0.3	8.2 (0.26-20.4) vs. 11.5 (4.0-43.3)	1.7 (0.8-3.8)	0.1	5.0 (0.26-17.5) vs. 6.4 (1.5-33.5)
ESCC	200c-3p	4-4	1.52 (0.4-6.3)	0.5	7.4 (3.7-9.7) vs. 5.8 (0.03-9.9)	1.0 (0.3-4.0)	1.0	6.0 (3.7-7.9) vs. 3.4 (0.03-8.7)
ESCC	375	4-4	1.5 (0.4-6.3)	0.5	6.6 (0.03-9.7) vs. 7.5 (1.9-9.9)	1.5 (0.4-6.3)	0.5	4.2 (0.03-7.9) vs. 6.0 (1.9-8.7)
ESCC	21-5p	4-4	0.4 (0.1-1.8)	0.1	9.6 (3.7-9.9) vs. 3.6 (0.03-9.7)	0.4 (0.1-1.8)	0.1	6.3 (3.7-8.7) vs. 3.3 (0.03-7.3)
ESCC	148a-3p	4-4	0.4 (0.1-1.8)	0.1	9.6 (3.7-9.9) vs. 3.6 (0.03-9.7)	0.4 (0.1-1.8)	0.1	6.3 (3.7-8.7) vs. 3.3 (0.03-7.3)



(a) (b)

Figure 3. (a) Kaplan-Meier analysis of OS in patients with GC in relation to plasma miR-200c-3p. N=5 in each group; p=0.06. (b) Kaplan-Meier analysis of OS in patients with EAC in relation to plasma miR-375. N=9 in each group; p=0.02.

EC and combined AC analysis

A significant relation was found between a high miR-375 expression and a better OS (14.9 vs. 10.1 months; HR=0.4 95% CI: 0.1 to 1.1, p=0.02) in patients with EC, while this was not observed for PFS (HR=0.5 95% CI: 0.2-1.3, p=0.1) (Table 3 and Figure 3b). No statistically significant relations were found between miR-200c-3p, miR-21-5p or miR-148-3p expression and either OS or PFS (Table 3). A combinatorial analysis of miR-375 combined with miR-21-5p or miR-200c-3p did not result in an increased significant relation to OS (data not shown). Since adenocarcinomas from patients with AC primary from GC and EC origin show similar molecular patterns [39] and for both circulating miR-200c-3p was determined, the analysis was also performed on these groups combined. However, miR-200c-3p was not related to OS or PFS in this group (Table 3).

ESCC

No significant relation was found between miR-200c-3p, miR-375, miR-21-5p or miR-148-3p expression and OS or PFS in patients with ESCC (Table 3).

miRNA expression at baseline and during chemotherapy in plasma of patients with GC and EC

Measurable baseline and follow-up samples were available of 7 patients with GC and 6 patients with EC (n= 4 AC; n = 2 SCC). miR-200c-3p (n=7), miR-146-5p

(n=7), miR-141-3p (n=2) and miR-218-5p (n=2) were measured in GC samples and miR-200c-3p (n=5), miR-375 (n=5), miR-21-5p (n=6) and miR-148a-3p (n=6) were measured in EC samples. No significant change in the expression of the measured miRNAs was observed between baseline and follow-up samples of patients with a favorable PFS compared with poor PFS, based on the median PFS (data not shown). Differences in miR-141-3p and miR-218-5p expression in GC samples could not be analyzed due to the small sample size.

miRNA relation with response to treatment in plasma of patients with GC and EC

For each miRNA the expression levels between patients with partial response (PR), or no response (stable disease, SD/progressive disease, PD) were compared. Three patients did not receive chemotherapy after randomization (1GC; 2EC). The baseline samples of these patients are not included in the response analysis but were included in the intention to treat analysis for PFS and OS. In GC no significant differences in ci-miRNA expression levels were observed between patients with PR or no response (SD/PD) (miR-200c-3p, n=4 vs. 5, p=1.0; miR-146a-5p, n=4 vs. 5, p=0.7; miR-141-3p, n=2 vs. 4, p=0.1; miR-218-5p, n=2 vs. 3, p=0.9). Also, in patients with EC (AC and ESCC) no significant differences in ci-miRNA expression levels were observed between patients with PR or no response (SD/PD) (miR-200c-3p, n=5 vs. 19, p=0.8; miR-21-5p, n=5 vs. 19, p=0.2; miR-375, n=5 vs. 19, p=0.4; miR-148a-3p, n=5 vs. 18, p=0.4), (data not shown). None of the patients had a complete response (CR).

miRNA expression and clinical benefit from palliative chemotherapy

The expression of miR-200c-3p in patients with AC primary from GC and EC origin was not different between patients with (n=14) or without (n=19) clinical benefit of palliative chemotherapy (n=33; HR=0.8 95% CI: 0.4 to 1.6, p= 0.6). Clinical benefit was defined as PR, CR or SD after 6 cycles vs patients with PD during chemotherapy.

Vitamin supplementation and miRNA expression

For the conducted clinical trial patients were randomized into treatment groups with or without vitamin supplementation. Differences in miRNA expression of the follow-up samples were compared between patients treated with chemotherapy supplemented with or without vitamin. Only for miR-146a-5p a trend towards a significant decreased miR-146a-5p expression in patients who were randomized

to vitamin supplementation was observed (median expression 0.8, range 0.2-1.2; n=3 vs 10.0, range 2.4-28; n=4; p=0.06). Differences in miR-141-3p and miR-218-5p expression in GC samples could not be analyzed due to the small sample size.

Discussion

The measurement of miRNAs in heparinized plasma samples is challenging. Heparin interferes with the detection of miRNAs by RT-qPCR [34]. Measurement of miRNA expression with ddPCR was managed despite storage in heparin over a longer period by using heparinase treatment to antagonize the effect of heparin. The optimal reagent contained 12 U heparinase with 30 µl RNA input. According to recent literature an endogenous normalization method was considered not necessary for ddPCR[40]. Cel-miR-39-3p was used as a control for technical variability. Adequate detection of cel-miR-39-3p was observed after a relatively high number of quantification cycles compared to fresh blood samples of the healthy donors (during the optimization experiment) and other publications[33]. The reason for this observation is not clear.

A significant relation was found between a high plasma miR-375 expression and a longer OS in patients with EAC treated with first-line palliative chemotherapy with gemcitabine and cisplatin. To our knowledge the prognostic value of circulating miR-375 in patients with EAC treated with palliative chemotherapy has not been shown before. High plasma miR-375 expression could indicate that tumors from these patients overexpress miR-375 although the plasma level may not reflect the level of microRNA in the tumor. Previous studies reported similar findings in tissue samples and cell lines, low levels of miR-375 were associated with a worse prognosis in tissue of Barrett associated EAC[41,42]. Upregulation of miR-375 in tissue and cell lines of pancreatic adenocarcinoma also inhibited cell growth and induced apoptosis, while the downregulation of miR-375 had the opposite effect. miR-375 may suppress the malignant behavior of pancreatic adenocarcinoma through decreased activation of the AKT signaling pathway[43]. An increased AKT pathway has been associated with resistance to several drugs, including cisplatin and gemcitabine. These drugs activate the AKT pathway which may act as a survival pathway[44]. Activating mutations of the AKT signaling pathway are also found in EAC[45]. The relation of miR-375 and gemcitabine and cisplatin is not

completely known. Upregulation of miR-375 increased the cisplatin-sensitivity of gastric cancer cells[46].

In our study, no significant relation was found between circulating miR-200c-3p, miR-21-5p or miR-148a-3p expression and OS or PFS in patients with EAC. Upregulated miR-21 in tissue of patients with EAC was previously associated with a worse prognosis[25] and resistance to gemcitabine treatment in other tumor types[26]. Upregulated miR-148 in tissue was also suggested to be associated with a worse prognosis in EAC[10]. In our study, no significant relation was found between circulating miR-375, miR-200c-3p, miR-21-5p or miR-148a-3p expression and survival in patients with ESCC. miR-200c, miR-148 and miR-375 were previously suggested to function as a tumour suppressor gene in ESCC [15,23]. A high baseline expression of miR-200c-3p showed a trend towards a worse OS of patients with GC in our study. Similar results were reported in earlier studies evaluating the function of miR-200c in GC, although it has been shown that a high miR-200c expression may activate the AKT pathway, leading to resistance to cisplatin[47]. High miR-200c levels in the blood of patients with GC were previously also associated with poor OS [13,15]. Interestingly Li et al. reported an association of higher miR-200c levels with better survival outcomes [16]. No significant relation between miR-141-3p, miR-146a-5p or miR-218-5p expression and survival in patients with GC were found in our study. Previous reports suggest that miR-141 and miR-146a may function as a tumour suppressor gene in GC [18,20,31]. miR-218 expression was found to be higher in GC cells sensitive for fluorouracil, oxaliplatin or adriamycine [48].The difference in results between our study and the previously reported results could be related to the fact that only a few of the available studies focused on patients that were treated with platinum containing chemotherapy for advanced EC and GC. The studies that reported miRNA measurements during chemotherapy were performed in Asian populations and focused on miR-200c-3p[16,23,24]. Unless optimisation of the heparinase treatment we cannot exclude an effect of the relatively old heparinized plasma samples that were used for analysis of miRNA samples in this study.

Twenty of the 38 included patients received supplementation with vitamin B12 and folic acid in addition to cisplatin and gemcitabine. We previously reported that the addition of folic acid and vitamin B12 did not improve RR, PFS or OS [28]. A folate deficient environment is associated with ***in vitro*** alteration of various

miRNAs[49]. The patients in this trial received folate supplementation in addition to a normal diet. In the current study, no significant differences were seen between miRNA levels in patients with or without vitamin supplementation although the sample size is small. In addition, it is difficult to draw conclusions from the measurements in the follow-up samples and analysis by response due to the small sample size. Current treatments of advanced GC and EAC generally consist of first-line fluoropyrimidine- and oxaliplatin based chemotherapy regimens either alone or, in case of Her2 overexpressing tumours, in combination with trastuzumab. Cisplatin and gemcitabine can still be a preferred treatment option in patients with preexisting neuropathy or dysphagia. After disease progression systemic therapy using paclitaxel and ramucirumab (for AC) and paclitaxel can be considered [50]. The role of miRNAs in relation to the response of these agents is not clear.

In conclusion assessment of miRNAs with ddPCR is possible in plasma samples despite prolonged storage in heparin. A better OS was seen in patients with EAC with a high plasma miR-375 expression compared to a low miR-375 plasma expression. No prognostic and/or predictive value was found for miR-21-5p, miR-148a-3p, miR-146a-5p, miR-141-3p and miR-218-5p in patients with advanced EC or GC treated with palliative cisplatin and gemcitabine but high levels miR-200c-3p showed a trend towards a worse OS.

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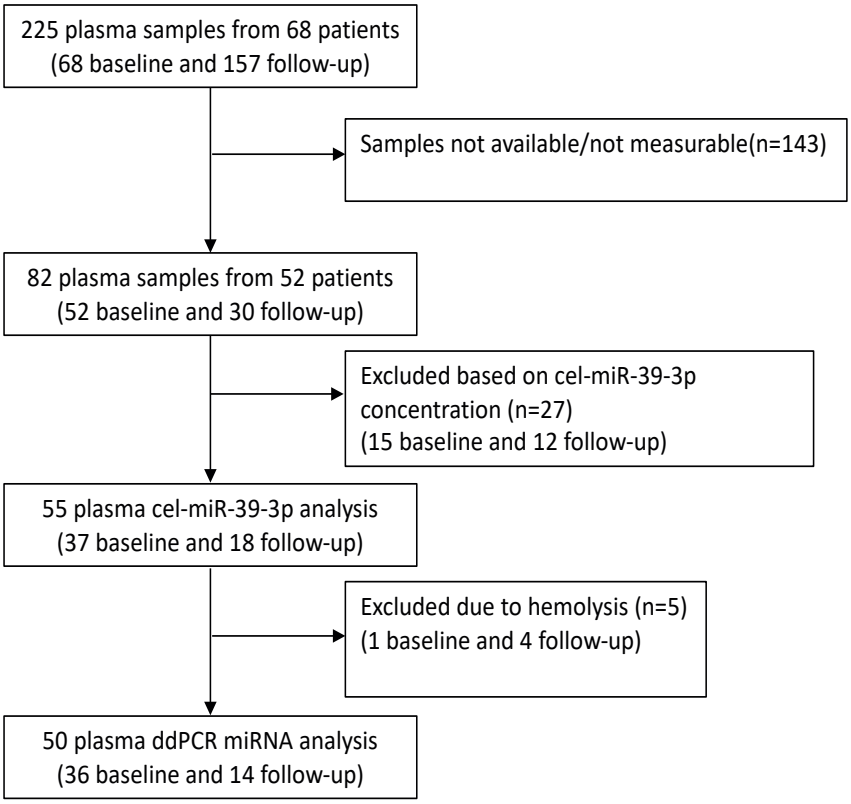
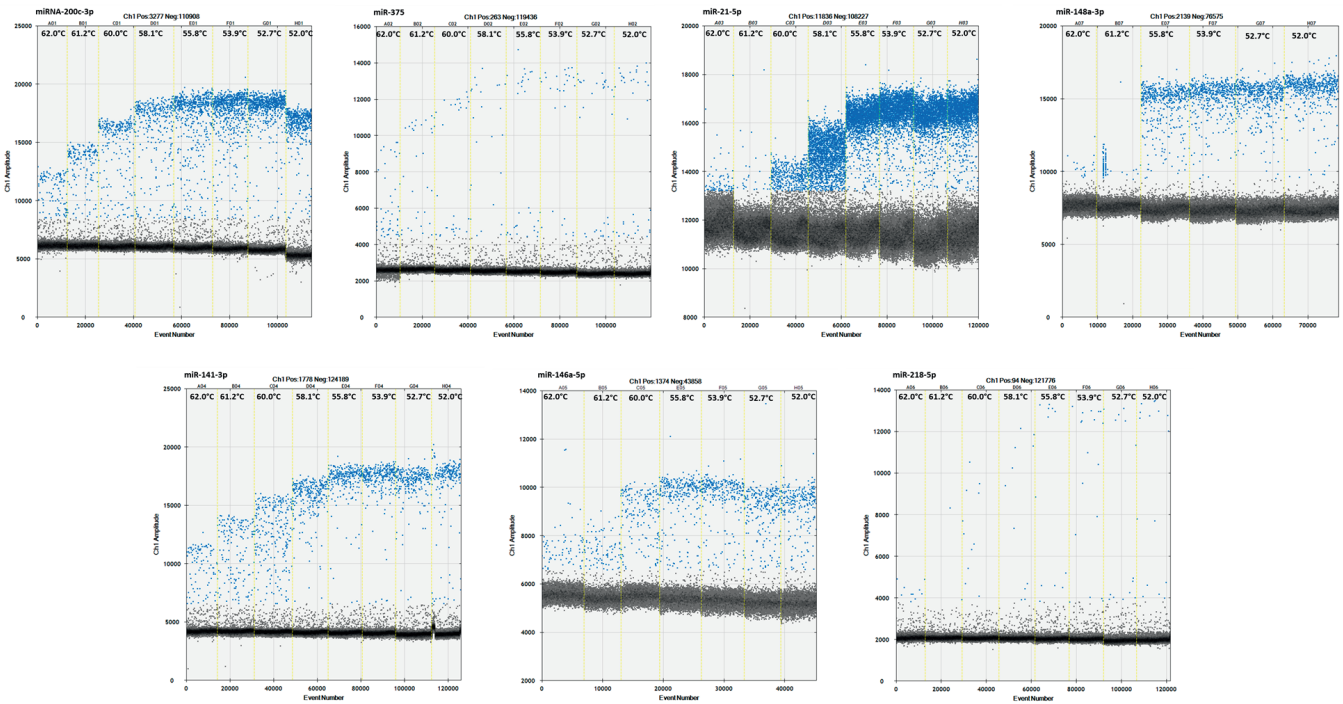
Supplementary Figures



Supplemental Figure S1: Flow chart of included samples

Supplemental Figure S2: Thermal gradient for primer assay optimization. The dd qPCR is performed at 8 different annealing temperatures: 62.0°C, 61.2°C, 60.0°C, 58.1°C, 55.8°C, 53.9°C, 52.7°C, 52.0°C. The following primers are used: miR-200c-3p, miR-375, miR-21-5p, miR-148a-3p, miR-141-3p, miR-146a-5p and miR-218-5p. Droplet are spaced out individually for fluorescence reading by the droplet reader. Positive droplets, which contain at least one copy of the measured miRNA exhibit increased fluorescence compared to negative droplets. Fluorescence in two channels is then measured for individual droplets. Each droplet from a sample plotted on the graph of fluorescence intensity vs. droplet number. The droplets in blue represent the positive droplets and the droplets in grey represent the negative ones. miR-148a-3p at 58.1°C is not visualized due an insufficient number of formed droplets (<10,000).



6

Summarizing discussion and future perspectives

This final chapter summarizes the findings and main conclusions of the reported studies in this thesis followed by an interpretation and discussion.

Part one: Pancreatic cancer

Chemoradiation and systemic therapy

In part 1 we focus on two clinical studies to improve the treatment of pancreatic cancer. As described in the introduction of this thesis only a minority of patients present with resectable disease. Resection alone typically results in a 5-year overall survival (OS) rate of approximately 10%. However, the prognosis for patients with resected pancreatic ductal adenocarcinoma (PDAC) is notably enhanced with the addition of adjuvant chemotherapy. Adjuvant chemotherapy with 5-fluorouracil (5-FU), folinic acid, irinotecan and oxaliplatin (modified FOLFIRINOX) vs gemcitabine improved median OS (53.5 vs 35.5 months) after 5 years follow up (1). The optimal neoadjuvant treatment with chemotherapy or chemotherapy in combination with radiotherapy (CRT) for patients with resectable and borderline resectable tumours is not clear and preferably given in a clinical trial. Neoadjuvant CRT with gemcitabine, followed by resection and 4 courses of adjuvant gemcitabine resulted in an 15% 5-year OS improvement versus immediate surgery and 6 courses of adjuvant gemcitabine in the PREOPANC study (patients with resectable or borderline resectable tumors) (2). The PREOPANC-2 trial compared FOLFIRINOX versus CRT with gemcitabine and adjuvant gemcitabine in the same neoadjuvant setting. Interestingly neoadjuvant FOLFIRINOX did not improve OS or resection rates compared to neoadjuvant gemcitabine based CRT (OS 21.9 vs. 21.3 months; HR 0.87; 95% CI 0.68-1.12, p=0.28; resection rates 77% vs. 75%, p=0.69)(3).

Around 35% of patients present with unresectable locally advanced pancreatic cancer (LAPC). Treatment with CRT in combination with gemcitabine has been extensively studied for LAPC and did not consistently show survival benefit compared to chemotherapy alone (4, 5). Human pancreatic cancer cells overexpress the epidermal growth factor receptor (EGFR) compared to normal pancreatic cells (6). Clinical data have shown a modest survival benefit by the addition of the EGFR inhibitor erlotinib to gemcitabine alone for patients with advanced PDAC (7). Therefore the combination of EGFR inhibitors cetuximab or erlotinib with gemcitabine based CRT was investigated in a pancreatic cancer model (8). In this

study EGFR signaling was examined by assessing phosphorylated EGFR and AKT protein levels in pancreatic cancer cell lines and tumor xenografts. The addition of an EGFR inhibitor to gemcitabine based CRT showed a reduction in EGFR and AKT protein levels and impaired tumor growth in tumor tissue compared to CRT with gemcitabine alone, indicating that the radiosensitizing effect of gemcitabine may be improved by EGFR inhibition. Several studies demonstrated that EGFR pathway inhibition in PDAC can improve the antitumor efficacy of RT independent of the KRAS mutation status of a tumor (9). This is relevant because KRAS mutations are common and associated with a worse prognosis in PDAC (10). Suggested mechanisms for EGFR inhibition regardless of downstream activating KRAS mutations are inhibition through the EGFR-PI3K-AKT pathway or HRAS signaling pathway (9, 11). Based on these preclinical data we investigated the safety, tolerability, and potential clinical efficacy of the human monoclonal anti-EGFR monoclonal antibody panitumumab (12) when added to CRT with gemcitabine in patients with LAPC. This phase I study is described in **chapter 2**. Patients diagnosed with LAPC and a WHO performance status of 0 to 1 were treated with panitumumab once a week at four different dose levels (1 to 2.5 mg/kg). This was combined with gemcitabine at a dose of 300 mg/m2 once a week along with radiotherapy (50.4 Gy in 28 fractions) during 6 weeks. Subsequently, patients received gemcitabine at a dose of 1,000 mg/m2 weekly for 3 weeks every 4 weeks, until either disease progression or unacceptable toxicity occurred. Each dose cohort was closely monitored during combination therapy to identify dose-limiting toxicity. Tumor assessment was carried out after CRT and during gemcitabine monotherapy. Fourteen patients were enrolled, with 14 being assessable for toxicity and 13 for response. The Maximum Tolerated Dose (MTD) for panitumumab in this combination was determined to be 1.5 mg/kg. Partial response was observed in 3 patients (23%), one in each dose cohort. Major toxicities potentially related to the combination of panitumumab and gemcitabine were nausea, vomiting, neutropenia, fatigue, and anorexia, whereas acneiform rash was considered to be definitively related to panitumumab. We concluded that adding panitumumab to gemcitabine-based CRT is feasible with considerable, but manageable toxicity. The median progression free survival (PFS) and OS of respectively 11.8 and 17.0 months in the MTD cohort and median PFS of 8.9 months for the three cohorts combined suggest some efficacy, although this cannot be established in a phase I study design with small numbers of patients.

Despite demonstrating the feasibility of this treatment and the observed relatively favorable PFS and OS, this phase I study was not succeeded by a phase II study. Meanwhile the standard chemotherapy with gemcitabine changed to multi-agent FOLFIRINOX after the PRODIGE-4 trial conducted by Conroy et al. in which FOLFIRINOX demonstrated an enhanced response rate (RR) and improved OS compared to gemcitabine monotherapy for patients with metastatic PDAC (13). The current guideline recommends induction chemotherapy with FOLFIRINOX as initial treatment also for patients with LAPC followed by an additional resection in selected patients. FOLFIRINOX for LAPC is associated with a median OS of approximately 12 months in a multicenter cohort study (14) and extends to 16-24 months in clinical trials (15, 16). Novel local therapies such as radiofrequency ablation (RFA) and irreversible electroporation (IRE; a nonthermal ablative technique using direct current) are used and studied extensively for the treatment of LAPC (17). Randomized studies in which local therapy is compared with systemic therapy are important to determine the most favorable treatment strategy. The efficacy of the addition of RFA to FOLFIRINOX chemotherapy is being investigated in the Pelican study (ClinicalTrials.gov Identifier: NCT03690323). The CROSSFIRE Trial compares FOLFIRINOX followed by IRE with FOLFIRINOX followed by stereotactic ablative radiotherapy (SABR) (ClinicalTrials.gov Identifier: NCT02791503). The results of these randomized studies are awaited for further treatment optimization. The concept of CRT with gemcitabine remains an interesting treatment option for future trials, particularly given the positive results of the PREOPANC trial. The addition of an EGFR inhibitor such as panitumumab to CRT may be explored in future trials considering the feasibility data of the phase I study described in **chapter 2**.

Current guidelines advise combination chemotherapy with FOLFIRINOX in patients with metastatic PDAC in a good clinical condition based on the PRODIGE-4 trial (13). Nab-paclitaxel plus gemcitabine can also be considered as initial systemic treatment, based on the results of the MPACT trial (median OS 8.5 vs. 6.7 months for gemcitabine monotherapy) in patients with metastatic PDAC (18). Nab-paclitaxel plus gemcitabine is often recommended for patients in a moderate condition or older age (>75 years) based on the eligibility criteria of these trials. First-line modified FOLFIRINOX and nab-paclitaxel plus gemcitabine regimens were recently compared in the planned interim analysis of 527 patients in the multicenter, randomized, open-label, phase II/III JCOG1611-GENERATE trial, as presented at the ESMO congress (October 2023, abstract 1616O) (19). The authors recommend nab-

paclitaxel plus gemcitabine as the first-line treatment for patients with metastatic or recurrent pancreatic cancer compared to modified FOLFIRINOX or S-IROX (S1 in combination with irinotecan and oxaliplatin) based on the primary endpoint, a median OS of 17.1 months with nab-paclitaxel plus gemcitabine, 14.0 months with modified FOLFIRINOX (HR 1.31), and 13.6 with S-IROX (HR 1.35). The study was terminated for futility since the predictive probability for achieving superiority at the final analysis was 0.73% for modified FOLFIRINOX and 0.48% the S-IROX. Grade 3–4 non-hematological toxicity (anorexia) rates were higher for modified FOLFIRINOX (23.3%) and S-IROX (27.5%) compared to nab-paclitaxel plus gemcitabine (5.0%). These results are markedly different compared to the PRODIGE-4 and MPACT trials and also to a previous Dutch nationwide cohort study (20). Peer reviewed publication of this trial is awaited to provide a better understanding of the factors contributing to the observed differences in toxicity profiles and treatment outcomes. FOLFIRINOX is the most commonly used first-line systemic treatment for patients with metastatic PDAC in the Netherlands (20). In the PRODIGE-4 trial, the median number of treatment cycles of first-line FOLFIRINOX administered was 10, with a range from 1 to 47 cycles (13). In daily clinical practice, the maximum number of cycles is commonly limited to 12 cycles in patients with stable disease or response followed by a chemotherapy interruption to reduce toxicity. Reintroduction of FOLFIRINOX can be considered when signs of disease progression occur after a 4-6 months treatment free interval since the last FOLFIRINOX administration. This approach is not supported by clinical studies. In **chapter 3** we describe a retrospective nationwide cohort study focusing on patients with advanced PDAC (not eligible for curative treatment) who were treated with FOLFIRINOX reintroduction after a therapy-free interval of 3 months or more following first-line palliative FOLFIRINOX. Data of the Netherlands Cancer Registry (NCR) were used for this cohort study. Out of the total 1381 patients who received first-line FOLFIRINOX, 119 were treated with FOLFIRINOX reintroduction after a therapy-free interval of 3 months or more. A favorable median OS of 23 months from diagnosis and 8.3 months from reintroduction of FOLFIRINOX with a median therapy-free interval of 7 months between first-line and FOLFIRINOX reintroduction was seen after FOLFIRINOX reintroduction. To our knowledge this is the first study that focuses on reintroduction of FOLFIRINOX after a treatment interruption following initial palliative FOLFIRINOX. Several studies explored a maintenance approach following induction chemotherapy with FOLFIRINOX or nab-paclitaxel plus gemcitabine. Maintenance therapy with 5FU, FOLFOX, or FOLFIRI after induction chemotherapy with FOLFIRINOX seems to be effective in patients with metastatic PDAC. (15-18). For patients with a known germline BRCA1/2

mutation olaparib can be considered after FOLFIRINOX based on PFS benefit in the POLO trial although no significant OS benefit was observed (21). The OS of patients retreated with FOLFIRINOX without maintenance therapy in our cohort does not seem detrimental compared to the survival rates in the maintenance studies, although these data cannot be directly compared. Drawing conclusions from our cohort study is challenging because of the diverse patient population found in a real-world cohort comprising all individuals who received systemic therapy for PDAC in the Netherlands. The database was based on information registered in the medical charts without strict inclusion criteria, uniform registration of given treatments and response monitoring such as used in clinical trials. We did not compare our patients with those who initiated a different second-line treatment because our primary focus was on patients who underwent retreatment with FOLFIRINOX, rather than those with FOLFIRINOX-resistant disease. The small subgroup of patients that was retreated with FOLFIRINOX experienced stable disease or response during first line FOLFIRINOX as best response and a favorable OS (with a large range in OS and treatment cycles). To further identify patients who could particularly benefit from this approach, we looked at characteristics of patients that either stopped or died 30 days after restarting FOLFIRINOX. In these patients (approximately 20% of the patients in which FOLFIRINOX was reintroduced), the therapy-free interval after first-line FOLFIRINOX was shorter (5.4 vs. 7.1 months) compared to patients who were treated > 30 days with FOLFIRINOX reintroduction.

Despite all the limitations of a retrospective cohort study, the results described in chapter 3 can be used as background information to provide answers of real world outcomes to patients who are eligible and consider retreatment with FOLFIRINOX. Also the knowledge that a drug holiday of many months is used in selected patients with reasonable outcomes can be useful.

The ideal second-line systemic therapy for patients who remain in good clinical condition after FOLFIRINOX failure (FOLFIRINOX resistant PDAC) remains uncertain. Retrospective data reported an OS of 11.5 months with gemcitabine-based therapy following FOLFIRINOX resistance (22). Liposomal irinotecan and 5-FU/leucovorin are approved for use in metastatic PDAC patients previously treated with gemcitabine-based therapy, based on improved median OS compared to 5-FU/LV alone in the NAPOLI-1 trial (OS 6.2 vs 4.2 months; HR 0.75) (23). A Dutch cohort study reported a median OS of 11.2 months with various second-line systemic therapies (20). Best supportive care is considered for patients in moderate condition.

Part two: oesophageal and gastric cancer

Systemic therapy and miRNAs.

In **chapter 4** we describe the results of a multicenter randomized open label phase 2 trial that aimed to improve the RR of palliative first-line chemotherapy for patients with advanced oesophageal cancer (EC) and gastric cancer (GC) by adding folic acid and vitamin B12 to the (at the time) commonly used schedule of cisplatin in combination with gemcitabine (24, 25). The rationale of this study consisted of an effect of the folate environment to cisplatin sensitivity of human cancer cell lines (26, 27) and the efficacy and toxicity benefits that were published after the addition of folic acid and vitamin B12 in a phase III study of pemetrexed in combination with cisplatin versus cisplatin in patients with a malignant mesothelioma as described in the introduction of this thesis (28). In the pemetrexed trial, a limited number of patients received chemotherapy without vitamin supplementation (in both the cisplatin and pemetrexed-cisplatin arm), and there was no randomization between those who received vitamin supplementation and those who did not. We first studied the sensitivity of adenocarcinoma cell lines for cisplatin under high and low folate conditions in the preclinical part of our study. It appeared that adenocarcinoma cells that were grown under high folate conditions were more sensitive to cisplatin and the intracellular platinum accumulation was higher in these cells, providing further support for the clinical part of the study in **chapter 4**. Patients with a histologically or cytologically confirmed metastatic or locally advanced EC (both squamous cell, ESCC or esophageal adenocarcinoma, EAC) or GC were included in this study. In the clinical part of the study a total of 82 patients were randomized from 2004 until 2013 between palliative treatment with cisplatin in combination with gemcitabine with or without folic acid and vitamin B12 suppletion. Vitamin suppletion did not result in a statistically significantly different RR (42.1% vs. 32.4%; $p = 0.4$). The median OS and time to progression (TTP) were 10.0 and 5.9 months, respectively, with chemotherapy and vitamin supplementation, compared to 7.7 and 5.4 months, respectively, with chemotherapy alone (OS, $p = 0.9$; TTP, $p = 0.9$). No difference in the plasma concentration of cisplatin and gemcitabine in the two treatment groups was observed, supporting the lack of a response benefit. The observation of lower plasma homocysteine levels in the supplemented group was interpreted as evidence of compliance with vitamin B12 and folic acid supplementation in the experimental group, since homocysteine is metabolized by folic acid and vitamin B12 metabolism (29). The different findings of the study in

chapter 4 compared to the pemetrexed study of Vogelzang et al. (used as rationale for our study) could potentially be explained by the different chemotherapy agents and a different trial design. In contrast to the pemetrexed study (28), our study was randomized for vitamin suppletion. Today, more options are available for the treatment of advanced EC and GC. Doublet schedules are preferred over triplet chemotherapy regimens, since the latter do result in more toxicity and only a limited survival benefit (30). First-line treatment with capecitabine and oxaliplatin (CAPOX) is presently the most commonly employed approach for patients with advanced or metastatic esophageal cancer (31, 32). Biomarker testing of the HER2 receptor is standard of care since the Toga trial found OS benefit for the addition of the HER2 antibody trastuzumab to palliative chemotherapy in advanced HER2-positive gastric adenocarcinoma or gastroesophageal junction tumors (33). A new treatment approach involving the humanized monoclonal PD-1 antibody pembrolizumab has been linked to an OS benefit (HR: 0.62; $p < 0.0001$), with a median OS of 13.5 months compared to 9.4 months when pembrolizumab is added to 5-FU and cisplatin in patients with HER2-negative advanced esophageal carcinoma or adenocarcinoma of the gastroesophageal junction, and high PD-L1 expression (CPS score > 10) (34). Dual PD-1 and HER2 blockade has also increased the response rate in interim analyses of the Keynote 811 trial in HER2-positive gastric or gastroesophageal junction adenocarcinoma (35). Furthermore there is more interest in the differences and similarities between ESCC and EAC since these types of cancer originate from the same organ but differ in genomic profile (36). Currently after disease progression second-line therapy using paclitaxel and ramucirumab and third line therapy with trifluridine/tipiracil can be considered for patients with an adenocarcinoma of esophagogastric origin in a good clinical condition (37, 38). In our study the use of second-line chemotherapy was not documented but was at the time very limited and unlikely to substantially confound our OS data. Although the trial described in **chapter 4** did not demonstrate a benefit with vitamin suppletion to palliative chemotherapy we want to point out some relevant aspects of the study. When we look at the chemotherapy backbone with cisplatin and gemcitabine in our phase 2 trial the median combined OS of 9.2 months and TTP of 5.4 months is comparable with current commonly used first-line palliative (doublet) chemotherapy regimens that are recommended for patients with advanced (CPS low) EC and GC. Cisplatin and gemcitabine are not used anymore in daily practice as first-line treatment, but these agents do have a different toxicity profile compared with now more commonly used 5-FU based chemotherapy regimens. Cisplatin and gemcitabine are in general

associated with more side effects compared to capecitabine and oxaliplatin, e.g. a higher incidence of grade 3 to 4 neutropenia, alopecia, thromboembolism, and renal dysfunction while peripheral neuropathy and diarrhea is a more frequent side effect of 5-FU and oxaliplatin (25, 31, 39, 40). Both 5-FU and its prodrug capecitabine are metabolized by the enzyme dihydropyrimidine dehydrogenase (DPD). Patients with a known impaired DPD activity can experience severe toxicity of 5-FU and capecitabine (41). Therefore, the here employed cisplatin and gemcitabine treatment combination may be potentially considered a palliative treatment option for selected patients who cannot tolerate 5-FU or oxaliplatin, although in daily practice cisplatin is often replaced by carboplatin to reduce toxicity. Another relevant topic of the study in **chapter 4** is the randomization of patients into two groups: one receiving vitamin supplementation and the other not. Mixed results and study designs are available for cancer prevention by vitamin B12 or folate supplementation (42, 43), and more recent studies show even an increased risk for lung or colorectal cancers by vitamin B12 supplementation (44, 45). To our knowledge there is no randomized evidence to support the use of folic acid or vitamin B12 in cancer treatment. Negative or neutral results from randomized trials, such as ours in **chapter 4**, can provide a counterbalance with reliable information to the popularity of vitamin use in cancer patients, which is often promoted without clinical evidence by social media (46-48).

In the current landscape of increased treatment options and increasing drug costs patient selection for the right treatment is very important to avoid unnecessary exposure of patients to toxic treatments and to enhance efficient use of healthcare costs. As described above important biomarkers like HER2 and PD-1 have been developed but these biomarkers require invasive tumor tissue analysis. Circulating microRNAs (ci-miRNAs) are extensively studied as potential noninvasive biomarkers for different tumor types. The definite role of miRNAs in EC and GC is unclear (49-51) and limited data are available about the function of miRNAs in plasma of patients with EC and GC who are treated with palliative chemotherapy. **Chapter 5** describes a study in which we investigated the prognostic and predictive value of ci-miRNAs measured by droplet digital PCR (ddPCR) within the plasma samples of patients treated with palliative chemotherapy for advanced EC or GC, as discussed in **chapter 4**. After conducting a literature search, miRNAs were chosen if evidence was reported in at least two independent publications. The selected miRNAs are as follows: for EAC and ESCC, miR-375, miR-200c-3p, miR-21-5p, and miR-148a-3p; for GC, miR-200c-3p, miR-141-3p, miR-146a-5p, and miR-218-5p. These ci-miRNAs were all known to be detectable in blood samples (52). A total of 225 plasma

samples stored in heparin were available of 68 patients (of the 82 patients who were included in the clinical study between 2004 -2013). From 63 patients a baseline sample was available (14GC; 49EC). A total of 53 follow up samples were available of these 63 patients (12GC; 41EC). Treatment of the samples with heparinase was required to counteract the known interaction between heparin and PCR analysis (53). Expression of ci-miRNA was analyzed in relation to overall survival (OS), progression-free survival (PFS), and response to chemotherapy. ci-miRNA levels were detectable in 36 baseline (71%) samples and in 14 (47%) follow-up samples. A significant relation was found between a high plasma miR-375 expression and a longer OS in patients with EAC treated with first-line palliative chemotherapy with cisplatin and gemcitabine, making this miRNA promising as a prognostic marker for patients with EAC. Increased circulating miR-200c-3p in GC showed a trend ($p = 0.06$) towards a shorter OS. Due to low evaluable sample numbers it was difficult to draw definitive conclusions from this study. No prognostic and/or predictive value was found for the other tested ci-miRNAs in contrast to previously reported literature as described in **chapter 5** reflecting the difficulty of reproducible results. No significant difference was observed in ci-miRNA expression between paired pre- and on-treatment samples and ci-miRNA expression was not associated with response to chemotherapy although numbers of available samples were low. While ci-miRNAs show promise as a non-invasive, stable biomarker with prognostic and predictive potential, their clinical application in daily practice requires reliable and precise data from extensive multicenter studies(54). Circulating tumor DNA is another noninvasive biomarker with promising results in different types of esophagogastric cancer(55). Further biomarker development is necessary to optimize patient selection to reduce toxicity of non-effective therapies and reduce treatment costs.

This thesis describes four different studies in patients with pancreatic cancer and esophagogastric cancer. The common goal of these studies was to improve the future prospects of patients with pancreatic cancer and esophagogastric cancer either by exploring the added effect of therapeutic interventions (panitumumab added to gemcitabine based CRT (**chapter 2**) and vitamin B12 and folate suppletion to gemcitabine-cisplatin (**chapter 4**), re-introduction of FOLFIRINOX chemotherapy after a therapy free interval (**chapter 3**)), and analyses of putative biomarkers for future patient selection (**chapter 5**). Despite recent progress in the field further studies are needed to advance the understanding and refine treatment strategies, ultimately improving the prognosis and outcomes for individuals diagnosed with pancreatic cancer and esophagogastric cancer.

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Addendum

Nederlandse samenvatting

Dit proefschrift geeft een overzicht van verschillende studies naar behandelingen en voorspellende biomarkers op het gebied van alvleesklierkanker en slokdarm-maagkanker.

In **hoofdstuk 1** wordt een algemene inleiding gegeven over de betreffende ziektebeelden waarbij de incidentie en verschillende behandelopties worden toegelicht.

Patiënten met een lokaal irresectabel pancreascarcinoom zonder metastasen op afstand (LAPC) of een pancreascarcinoom met metastasen op afstand komen doorgaans niet in aanmerking voor een curatieve behandeling. In plaats daarvan wordt palliatieve chemotherapie aanbevolen om de overleving te verbeteren en tumor gerelateerde symptomen te verlichten. Voor patiënten met LAPC kan, afhankelijk van de respons op inductie chemotherapie, de mogelijkheid van resectie opnieuw worden overwogen. Andere lokale behandelingen, zoals radiofrequente ablatie (RFA) of irreversibele electroporatie (IRE), worden doorgaans alleen in studieverband toegepast. Voor patiënten met LAPC of gemetastaseerde ziekte zijn FOLFIRINOX en gemcitabine met nab-paclitaxel momenteel veelgebruikte behandelingen, waarbij patiënten in goede conditie volgens de Nederlandse richtlijn bij voorkeur behandeld worden met FOLFIRINOX. In **hoofdstuk 2** werd de toevoeging van een antilichaam gericht tegen de epidermale groeifactor receptor (EGFR) aan een behandeling met radiotherapie in combinatie met het chemotherapeuticum gemcitabine onderzocht bij patiënten met een LAPC. Ten tijde van dit onderzoek werd radiotherapie in combinatie met chemotherapie (CRT) beschouwd als een standaard behandeling voor LAPC. De toevoeging van een EGFR remmer (panitumumab) was gebaseerd op preklinisch onderzoek waarbij blokkade van de EGFR gemedieerde signaaloverdracht in tumorcellen de gevoeligheid van pancreastumorcellen voor CRT verhoogde. Onze studie betrof een fase 1 studie waarin de klinische belasting en bijwerkingen van verschillende panitumumab doseringen in combinatie met op gemcitabine gebaseerde CRT werd onderzocht. De maximale verdraagbare dosis (MTD) van panitumumab werd vastgesteld op 1,5 mg/kg. We concludeerden dat het toevoegen van panitumumab aan CRT met gemcitabine haalbaar is met aanzienlijke, maar te behandelen bijwerkingen. Veel voorkomende bijwerkingen waren misselijkheid, braken, neutropenie, vermoeidheid en anorexie en acneïforme huiduitslag. Deze klachten zijn behoudens huiduitslag ook te beschouwen als potentieel ziekte-

specifieke symptomen. Acneïforme huiduitslag is een bekende bijwerking van panitumumab. Hoewel de overlevingsresultaten met een mediane progressievrije overleving (PFS) van 11,8 maanden en een algehele overleving (OS) van 17,0 maanden in het MTD-cohort, enige werkzaamheid van deze behandeling suggereren, kan de effectiviteit van deze behandeling in een fase 1 studie-opzet met een klein aantal patiënten niet worden vastgesteld.

In **hoofdstuk 3** wordt een landelijk cohortonderzoek beschreven wat is gericht op patiënten met een irresectabel pancreascarcinoom (met of zonder metastasen) die werden behandeld met 1e lijns combinatie chemotherapie in de vorm van FOLFIRINOX. De data voor dit onderzoek werden verkregen uit de Nederlandse Kankerregistratie (NKR). In de oorspronkelijke studie van Conroy et al. in 2011 waarin de effectiviteit van FOLFIRINOX is aangetoond bedroeg het mediane aantal behandelingscycli 10 (range 1-47 kuren). In de dagelijkse praktijk wordt het maximale aantal cycli bij patiënten met stabiele ziekte of respons doorgaans beperkt tot 12 om hevige bijwerkingen te verminderen en is herintroductie van FOLFIRINOX gebruikelijk wanneer er ziekte progressie optreedt na een behandelpauze van minimaal 4-6 maanden. Wetenschappelijke onderbouwing voor deze aanpak ontbreekt echter. In **hoofdstuk 3** onderzochten we hoe vaak deze herintroductie van FOLFIRINOX in Nederland wordt toegepast en keken we naar de uitkomsten van deze behandeling om zo de meest geschikte patiëntencategorie voor deze behandelstrategie te identificeren. Een klein gedeelte van de patiënten die aanvankelijk behandeld werd met FOLFIRINOX kreeg opnieuw FOLFIRINOX chemotherapie na een behandelvrije periode van minimaal 3 maanden. Deze herintroductie van FOLFIRINOX bleek het meest gunstig te zijn voor patiënten die een goede respons en beperkte bijwerkingen hadden gehad tijdens de eerste behandelperiode, wat zich vertaalde in een langer chemotherapievrij interval (ongeveer > 6 maanden). Hoewel er beperkingen zijn aan een retrospectieve cohortstudie, kunnen deze resultaten, die gebaseerd zijn op de dagelijkse praktijk, behulpzaam zijn als achtergrond informatie wanneer wordt overwogen om FOLFIRINOX opnieuw in te zetten.

In **hoofdstuk 4** worden de resultaten van een gerandomiseerd multicenter fase 2 onderzoek beschreven. Dit onderzoek had als doel om de respons op eerstelijns palliatieve chemotherapie bij patiënten met gevorderde slokdarm-maagkanker te verbeteren door foliumzuur en vitamine B12 aan de chemotherapie doublet

cisplatin en gemcitabine toe te voegen. De rationale voor dit onderzoek bestond uit een verhoogde effectiviteit en verminderde toxiciteit die werden gerapporteerd na toevoeging van foliumzuur en vitamine B12 aan pemetrexed in combinatie met cisplatin versus cisplatin monotherapie in een fase III-studie bij patiënten met een mesothelioom. In het preklinische deel van het onderzoek in **hoofdstuk 4** werd in vitro inderdaad een hogere gevoeligheid van adenocarcinoom cellen voor cisplatin gevonden na toevoeging van foliumzuur wat ondersteuning bood voor het klinische gedeelte van deze studie. Er werd in het klinische fase 2 gedeelte van de studie in **hoofdstuk 4** echter geen verhoogde respons op chemotherapie of overlevingsvoordeel gezien na vitamine suppletie. Dit gebrek aan verbetering in een gerandomiseerde gecontroleerde studie naar het gebruik van vitamines tijdens behandeling tegen kanker is relevant voor patiënten die overwegen in deze situatie vitamines te gebruiken. Deze studie geeft ook relevante informatie over het chemotherapieschema met cisplatin in combinatie met gemcitabine, wat nog steeds kan worden overwogen in specifieke omstandigheden.

In de huidige tijd van toegenomen behandelingsmogelijkheden en stijgende zorgkosten is de selectie van de juiste behandeling voor patiënten heel belangrijk om bijwerkingen van onvoldoende werkzame behandelingen te voorkomen en efficiënt gebruik van zorgkosten te bevorderen. Voorspellende biomarkers kunnen gebruikt worden om patiënten te selecteren voor de juiste behandeling. MicroRNA's (miRNAs) zijn kleine stukjes niet-coderend RNA die genexpressie kunnen reguleren door binding aan bepaalde delen van het messenger RNA. Hierdoor kunnen genen in meer of mindere mate tot expressie komen wat kan leiden tot tumorgroei of juist onderdrukking van tumorgroei. De aanwezigheid van verschillende miRNAs kan worden gedetecteerd in weefsels en bloed en is uitgebreid bestudeerd als potentiële biomarker voor verschillende tumortypen. De rol van miRNAs bij patiënten met een gemetastaseerd slokdarm of maagcarcinoom is nog onduidelijk en ook zijn er beperkte gegevens beschikbaar over de functie van miRNAs in het bloed van patiënten die met palliatieve chemotherapie worden behandeld voor een slokdarm of maagcarcinoom. In **hoofdstuk 5** beschrijven we een studie die zich richt op de prognostische en voorspellende waarde van miRNAs in plasmamonsters van patiënten die werden behandeld in het onderzoek beschreven in **hoofdstuk 4** met behulp van een recent geïntroduceerde zeer nauwkeurige kwantificatiemethode (droplet digital PCR). Er werd een significant verband gevonden tussen een betere overleving en een hoge expressie van

plasma miR-375 bij patiënten met adenocarcinoom uitgaande van de slokdarm. De andere onderzochte miRNAs vertoonden geen significante prognostische of voorspellende waarde. Zowel de positieve als negatieve resultaten van deze studie zijn nuttig voor toekomstige miRNA-onderzoeken. Daarnaast is deze studie is ook relevant omdat we een methode hebben ontwikkeld om miRNAs met behulp van droplet digital te detecteren in plasma na langdurige opslag in heparine.

Ondanks recente ontwikkelingen is er meer onderzoek nodig naar nieuwe behandelingen, het verbeteren van bestaande behandelstrategieën en optimalisatie van patiënt selectie, met als doel de vooruitzichten van patiënten met alvleesklierkanker en slokdarm-maagkanker te verbeteren.

Publications

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Curriculum vitae

Curriculum vitae

Annette van Zweeden was born on 31th July 1980 in the Hague, the Netherlands. She grew up in the Hague. She moved to Amsterdam after graduating from gymnasium Haganum in 1998 and studied medicine at the University of Amsterdam. She obtained her medical degree in 2005, following an internal medicine internship in Cape Town South Africa and a scientific internship at the AMC (now Amsterdam UMC, location AMC), where she studied hypertension and genetic variations across different ethnic populations under the supervision of prof.dr. R. P. Koopmans. She completed her junior internship at the AvL in Amsterdam, where her interest in oncology formed. After her medical degree she had the chance to start as a resident-not-in-training at the internal medicine department of the AMC. At that time her preference for internal medicine was already clear and she started her residency in internal medicine in 2006 in the AMC (supervision: prof. dr. P. Speelman). During this residency she worked also in the Westfries Gasthuis (now Dijklander) in Hoorn (supervision: dr. W.G. Meijer) and the OLVG (supervision: dr. W. Terpstra) in Amsterdam. She completed her subspecialty training in medical oncology at the AMC (supervision prof. dr. D. Richel and dr. Westermann) and AvL (supervision prof. dr. S. Rodenhuis). She started as a medical oncologist in the Maasstad Hospital in Rotterdam after her registration in 2012. In 2013 she returned to Amsterdam and worked as a medical oncologist and later PhD candidate in the VU University Medical Center (VUmc; now Amsterdam UMC, location VUmc) and Ziekenhuis Amstelland in Amstelveen. She combined her doctoral research with her role as an internist-oncologist at Ziekenhuis Amstelland. She is chair of the oncology committee and the medication committee at Ziekenhuis Amstelland, local principal investigator of various studies and active in regional tumour networks.

Annette is married to Joris Hogewind and they have three children: Sebastiaan (2012), and twins Max and Sabine (2015). They live in Haarlem.

Dankwoord

Dankwoord

Na een promotietraject van 10 (!) jaar ben ik erg blij en voldaan dat mijn proefschrift nu af is. Dat het gelukt is heb ik aan velen te danken. Ongetwijfeld zal ik mensen vergeten, maar ik zal mijn best doen jullie een beeld te geven van iedereen die mij heeft bijgestaan.

In 2014 ben ik begonnen met mijn promotie traject. Ik was toen net een half jaar werkzaam in het VUmc na mijn opleiding tot internist oncoloog in het AMC en een jaar chef de clinique in het Maasstad Ziekenhuis in Rotterdam. Mijn nieuwe baan voelde als de hoofdprijs. Ik werkte als internist-oncoloog in het VUmc, waarbij ik drie dagen per week gedetacheerd werd naar Ziekenhuis Amstelland en één dag per week de gelegenheid had om in het VUmc te werken aan het versterken van de samenwerking op het gebied van oncologische zorg tussen Ziekenhuis Amstelland en het VUmc. En dat alles op fietsafstand, niet onbelangrijk met een baby van nog geen jaar thuis. Nadat de VUmc-studies in Ziekenhuis Amstelland waren gestart, de protocollen waren afgestemd en de gezamenlijke multidisciplinaire overleggen waren opgezet, kreeg ik de kans om in het VUmc verder te gaan met wetenschappelijk onderzoek, hetgeen heeft geleid tot dit proefschrift.

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