Circulating Biomarkers in Pancreatic Cancer Patients Treated With FOLFIRINOX

To treat or not to treat?

Fleur van der Sijde

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To treat or not to treat?

Circulerende biomarkers in patiënten met pancreascarcinoom bij behandeling met FOLFIRINOX

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Chapter 1

Introduction and outline of this thesis

GENERAL INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and associated with a poor prognosis.[1] Five years after diagnosis only approximately 7% of PDAC patients will still be alive, which is significantly lower compared to the more frequent cancers, such as breast and prostate cancer with survival rates over 90%.[2-4] Although PDAC is a rare disease, its incidence is rising and due to the high mortality rate PDAC is expected to become the second leading cause of cancer-related death by the year 2030.[3]

There are several reasons for the lethality of PDAC. First of all, most patients lack symptoms and therefore present themselves at an advanced stage of disease when curation is no longer possible.[5-7] Second, PDAC shows aggressive behavior; tumors grow fast and metastasize very easily.[4, 8] Third, PDAC tumors respond poorly to the available treatment options.[7, 9-11]

Only 20% of patients is diagnosed with localized, resectable disease.[6, 7] Those patients are still eligible for surgical resection with curative intent. In the majority of patients, however, the disease is already disseminated and recurrence would develop shortly after surgery. For those patients, with locally advanced (LAPC) and metastatic PDAC, chemotherapy with or without radiation is currently the standard treatment, as more effective therapies are still lacking.[11, 12] FOLFIRINOX, a chemotherapy combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin, is the first-line treatment of choice because of its superior survival benefit compared to other chemotherapy regimens, such as gemcitabine-based treatment.[11-13] However, more than 60% of patients will experience FOLFIRINOX-related toxicity, with great impact on their quality of life.[10, 11, 14] Furthermore, not all patients respond to FOLFIRINOX. In 15-40% of patients, the tumor will grow or even metastasize already during chemotherapy.[10-12] Apparently, in these patients FOLFIRINOX has no, or only limited, cytotoxic effect on most cancer cells.

The aggressive behavior and lack of treatment response in PDAC are not completely understood. Genetic, epigenetic, and immunologic factors might contribute to the development and progression of the disease, which is a major subject in PDAC research, including the research described in this thesis.

PDAC tumors are surrounded by a dense stroma, rich in tumor-promoting (immune) components that support cancer cell proliferation.[15] It also prevents the infiltration of immune cells and penetration of therapeutic drugs into the tumor. Next to that, PDAC cells originate from pancreatic cells with genetic mutations that cause dysregulation of

many important cell pathways and induce uncontrolled cell growth.[16] The effect of DNA damage by chemotherapy or radiation, for example, can be actively repaired in cancer cells, and transporter proteins can eliminate toxic drugs from the cell through which the efficiency of chemotherapy is diminished.[17-19]

Genetic, epigenetic, and immunologic factors can be measured, for example, in tissue or blood samples. Many so-called biomarkers, measurable characteristics as indicators of physiologic and pathologic processes, have been found to associate with disease progression, treatment response, and prognosis in PDAC.[20, 21] For example, PDAC patients with tumor mutations in the *KRAS*, *TP53*, or *SMAD4* gene show shorter survival compared to patients with normal, wild-type *KRAS*, *TP53*, or *SMAD4* genes.[22-25] There are also indications that these tumors respond poorly to (chemotherapy) treatment, due to the aggressiveness of the tumors and escape mechanisms induced by upregulation of important cell pathways.[22, 25] It is also known that non-coding RNAs, e.g. microRNAs and long non-coding RNAs, have great influence on the function of genes and the proteins they code for. MicroRNAs for instance, regulate post-transcriptional gene expression; they can turn genes on and off, affecting the pathways in which these genes are involved. Dysregulation of these pathways can initiate insensitivity to chemotherapy-induced apoptosis, increase DNA repair mechanisms, dysregulate the cell cycle, and enhance multidrug resistance.[19, 26] Biomarkers can illuminate the unique biological behavior of tumors and potentially predict how the disease will develop over time.[20, 21, 27] Biomarkers could therefore be used to inform patients about their prognosis or side effects that they can expect from treatment. Predictive biomarkers for treatment response could be used to stratify patients for available therapies.[20] Moreover, investigating specific tumor characteristics might also help discover new therapeutic options.

Until now, the majority of PDAC biomarker research has been performed using tumor tissue. However, liquid biopsies, for example blood, serum, plasma, bile, and urine, have many advantages over tissue biopsies.[27, 28] Tissue material is often not available for research and tissue biopsies require an invasive, hazardous procedure, which is certainly not ideal when collecting several samples over time. Liquid biopsies on the other hand are relatively cheap, have little risks for the patient and can be obtained repeatedly. Also, circulating biomarkers from liquid biopsies represent a complete molecular make-up of a tumor and its occult metastases, whereas intratumoral and intertumoral heterogeneity cannot be reflected by a single tumor biopsy.[27, 28]

The main focus of this thesis is to investigate circulating biomarkers that can predict early tumor progression during FOLFIRINOX. Such predictive biomarkers for FOLFIRINOX response would enable patient stratification and prevent non-responding patients

from FOLFIRINOX-induced toxicity. These patients may benefit from other therapeutic options instead.

OUTLINE OF THIS THESIS

In **Part I** of this thesis, we investigate the response rate and survival of PDAC patients treated with FOLFIRINOX in the Netherlands. In addition, the quality of life after completion of FOLFIRINOX treatment in patients with locally advanced pancreatic cancer is assessed. In the second and third part of this thesis several biomarkers are investigated for their value to predict FOLFIRINOX response and the association of these biomarkers with survival of PDAC patients. In **Part II** we focus on circulating DNA mutations originating from tumor cells (ctDNA) that can be detected in blood plasma, and circulating microRNAs, which are important regulators of gene expression and cell pathways. In **Part III** we study immunological biomarkers, including circulating cytokines, for their predictive and prognostic value. Furthermore, we investigate what type of source of immune cells, stabilized whole blood or isolated peripheral blood mononuclear cells (PBMCs), is the best to use for immunologic gene expression analysis. Finally, the results from this thesis and future perspectives are discussed, followed by a Dutch summary.

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Part I

FOLFIRINOX treatment in clinical practice

Chapter 2

Treatment response and conditional survival in advanced pancreatic cancer patients treated with FOLFIRINOX: a multicenter cohort study

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ABSTRACT

Background FOLFIRINOX chemotherapy is the current standard of care for locally advanced (LAPC) and metastatic pancreatic cancer (PDAC). The objective of this study was to evaluate real-world FOLFIRINOX response rates and survival outcomes in advanced PDAC, and to assess conditional survival after completion of FOLFIRINOX chemotherapy.

Methods This was a multicenter, retrospective cohort study conducted in four hospitals in the Netherlands. Consecutive patients diagnosed with LAPC or metastatic PDAC, treated with FOLFIRINOX, were included.

Results Between 2012 and 2018, a total of 284 patients were included, of whom 136 were diagnosed with LAPC and 148 with metastatic PDAC. Objective response rates were similar in both groups: 14.0% in LAPC, and 18.2% in metastatic patients. The disease control rate was higher in LAPC patients (77.2%) compared to patients with metastatic PDAC (51.4%, $P<0.001$). The median overall survival (OS) of those with LAPC was 12.7 months (95% CI 11.4-14.1 months). Their probability of 2-year survival increased from 14% to 26% one year after completion of FOLFIRINOX. The median OS in patients with metastatic PDAC was 8.1 months (95% CI 6.5-9.6 months); the 2-year survival probability increased from 10% to 29% after one year.

Conclusions Our study provides real-world estimates of response rates, survival, and conditional survival in patients with advanced pancreatic cancer treated with FOLFIRINOX. These results are useful for patient counseling and for clinical decision-making.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a cancer known for its poor prognosis. Eighty percent of patients present with advanced disease at the time of diagnosis.[1] These patients are not eligible for surgical resection of the tumor, and therefore systemic chemotherapy is one of few treatment options. FOLFIRINOX chemotherapy (a combination of fluorouracil, leucovorin, irinotecan and oxaliplatin) is the current standard-of-care in the Netherlands. Although FOLFIRINOX has been associated with improved survival compared to other regimens,[2, 3] response rates still are disappointing,[4] and patients and physicians are sometimes reluctant to start FOLFIRINOX because of the high toxicity rate.[2, 5] However, reliable or useful data on response rates and survival benefit are lacking; most studies in the literature report data on metastatic patients only, or report outcomes for all disease stages combined, which are difficult to interpret for patient counseling. Moreover, patient outcomes reported from (randomized) clinical trials might be biased by patient selection before study entry. Therefore, real-world data are necessary to properly inform patients about expected chemotherapy outcomes and enable shared decision-making. Similarly, it is important to be able to inform patients about their future perspectives, also after completion of treatment. Survival probabilities might shift considerably during follow-up, for example after completion of FOLFIRINOX.

Conditional survival (CS) is the survival probability of patients who have already survived a certain period. CS is most relevant in the assessment of patients with cancers associated with high mortality rates early after diagnosis, such as PDAC. Prognosis is disproportionately negatively influenced by early deaths.[6, 7] Sharing personalized survival probability estimates are important to share with patients and may impact their future decision-making, quality of life, and mental well-being.[6]

The objective of this study was to evaluate real-world outcomes of FOLFIRINOX response and survival in advanced PDAC patients and to assess CS, using data from a multicenter, retrospective cohort study.

METHODS

Study design

This multicenter, retrospective study was conducted at four hospitals in the Netherlands: the Erasmus University Medical Center (Rotterdam), Amsterdam University Medical Center (Amsterdam), Maasstad Hospital (Rotterdam), and Franciscus Gasthuis (Rotterdam/Schiedam).

Patient selection

By searching hospital pharmacy records, we identified all consecutive patients who had received FOLFIRINOX chemotherapy for LAPC or metastatic PDAC between January 2012 and December 2018. Pancreatic malignancy was histologically confirmed in all patients. Locally advanced PDAC was defined according to the Dutch Pancreatic Cancer Group criteria; LAPC was defined as >90 degrees of arterial contact or >270 degrees of venous contact.[8] Patient characteristics such as age, sex, stage of disease, FOLFIRINOX chemotherapy specifics, laboratory results, CT scan evaluations, and follow-up data were retrieved from medical records. The study protocol was approved by the medical ethics review boards of all participating hospitals.

FOLFIRINOX treatment

Patients received FOLFIRINOX chemotherapy every two weeks in the following dosages: oxaliplatin 85 mg/m2 infused over 120 min, immediately followed by leucovorin at a dose of 400 mg/m2 infused over 120 min with the addition, after 30 min, of irinotecan 180 mg/m2 infused over 90 min, followed by a fluorouracil 400 mg/m2 intravenous bolus, followed by 2400 mg/m2 continuous infusion for 46 hours. FOLFIRINOX chemotherapy was discontinued if progression of disease was evident, at the patient's request, or in case of unacceptable toxicity.

Locally advanced patients were scheduled for eight to twelve cycles of FOLFIRINOX chemotherapy. Patients showing complete or partial response or stable disease after chemotherapy might have received stereotactic radiation therapy and/or surgical resection. Some patients participated in the PELICAN trial (Dutch trial register NL4997), in which stage of disease is re-evaluated after four cycles of FOLFIRINOX. In this trial, if there is no disease progression after four cycles of FOLFIRINOX patients are randomized between radiofrequency ablation followed by continuation of chemotherapy, or chemotherapy only. In both arms a total of 12 cycles of FOLFIRINOX is given. The standard of care in the Netherlands for metastatic disease patients is twelve cycles of FOLFIRINOX chemotherapy.

Treatment response evaluation

A CT scan to evaluate the tumor response to treatment was performed after every fourth cycle of FOLFIRINOX or earlier if patients showed clinical signs of tumor progression or treatable disease symptoms, such as biliary obstruction, that could be diagnosed with CT scans. The diameters of target lesions were determined by experienced radiologists, and treatment response was reported according to the Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria. Final response was defined as the treatment response measured on the latest available evaluation CT scan. The objective response rate (ORR)

was defined as the proportion of patients with complete response or partial response to treatment. The disease control rate (DCR) was defined as the proportion of patients with complete response, partial response, or stable disease.

Statistical analyses

Categorical patient characteristics (e.g., sex, location of the tumor, and RECIST response outcomes) were compared between LAPC and metastatic disease patients with Pearson's Chi-squared tests. Continuous patient characteristics (e.g., age, baseline CA 19-9, and number of FOLFIRINOX cycles) were not normally distributed and were therefore compared with Mann-Whitney U tests.

Overall survival (OS) was calculated as the time between start of chemotherapy and death, progression-free survival (PFS) as the time between start of chemotherapy and radiologic or histologic confirmation of disease progression. Differences in survival between LAPC and metastatic disease patients and between the different RECIST response outcome patient groups were estimated with Kaplan-Meier curves and compared with log-rank tests.

Conditional survival (CS) was defined as the probability that a patient would survive an additional number of months or years after already having survived a certain time. CS was calculated using Kaplan-Meier survival estimates with the formula: $CS_{(x/y)} = S_{(x/y)}/S_{(x)}$, with S = survival estimate, x = number of months survived and y = number of additional months. For example, to estimate the CS for surviving one additional year for patients who already had survived one year, $CS_{(12/12)}$ is calculated by dividing the 2-year Kaplan-Meier survival estimate $S_{(24)}$ by the 1-year Kaplan-Meier survival estimate $S_{(12)}$.[7, 9, 10]

All tests were performed two-sided, and *P*-values <0.05 were considered statistically significant. Data were analyzed using SPSS (version 25.0; IBM, Armonk, NY, USA) and R software, version 4.04

RESULTS

Patient characteristics

Data of 284 consecutive patients – 136 diagnosed with LAPC and 148 with metastatic PDAC – who started FOLFIRINOX were included in the analyses.

Patient characteristics are reported in Table 1. Median serum CA 19-9 levels prior to start of FOLFIRINOX in patients with metastatic PDAC was 919 kU/L (IQR 125-5622 kU/L)

and in LAPC patients 314 kU/L (IQR 92-1131 kU/L, *P*<0.001). In patients with metastatic PDAC, the primary tumor was more often located in the tail of the pancreas (35 patients [23.6%] vs 8 patients [5.9%], *P*<0.001). In the metastatic patient group, 122/148 patients (82.4%) showed metastases at PDAC diagnosis; the other 26 patients (17.6%) received FOLFIRINOX chemotherapy upon metastasis after surgical resection of the primary tumor. Of those patients, 15 showed local recurrence at the surgical resection site. Twenty-five patients, initially identified as having LAPC, were found to have metastatic disease on diagnostic laparoscopy. The median number of FOLFIRINOX cycles received did not significantly differ between the LAPC and metastatic PDAC groups. The majority of LAPC patients (71.3%) received additional treatment (e.g. stereotactic radiation, surgery, other chemotherapy, immune therapy) after FOLFIRINOX chemotherapy, whereas only 34.5% of metastatic patients received additional treatment (*P*<0.001). In this cohort, the resection rate in LAPC patients was 24/136 (17.8%). Eight of the resected patients received adjuvant gemcitabine chemotherapy. None of the patients with metastatic disease underwent surgery.

Table 1. Baseline and treatment characteristics of patients with locally advanced (LAPC) and metastatic pancreatic cancer.

	All patients *n*=284 (%)	**LAPC *n*=136 (%)**	**Metastatic disease *n*=148 (%)**	***P***
Age, median (range)	62 (31-81)	63 (37-80)	61 (31-81)	0.507
Age >75 years	51 (18.0)	27 (19.9)	24 (16.2)	0.375
Sex, male	151 (53.2)	69 (50.7)	82 (55.4)	0.431
Baseline CA 19-9 level (kU/L), median (IQR)	457 (105-2044) (*n*=220)	314 (92-1131) (*n*=105)	919 (125-5622) (*n*=115)	<0.001
Location primary tumor				
Head	173 (60.9)	94 (69.1)	79 (53.4)	<0.001
Body	67 (23.6)	33 (24.3)	34 (23.0)	
Tail	43 (15.1)	8 (5.9)	35 (23.6)	
Multifocal	1 (0.4)	1 (0.7)	0 (0)	
Recurrent disease after surgery	26 (9.2)	NA	26 (17.6)	NA
Local recurrent disease after surgery	15 (5.3)	NA	15 (10.1)	NA
Diagnostic laparoscopy prior to start FOLFIRINOX	114 (40.1)	89 (65.4)	25 (16.9)	<0.001
Number of FOLFIRINOX cycles, median (IQR)	8 (4-8)	8 (4-8)	7 (3-10)	0.639
Additional therapy after FOLFIRINOX	148 (52.1)	97 (71.3)	51 (34.5)	<0.001
Surgical resection after FOLFIRINOX	24 (8.5)	24 (17.8)	0 (0)	NA
Adjuvant chemotherapy after surgical resection	8 (2.8)	8 (33.3)	NA	NA

CA 19-9 = carbohydrate antigen 19-9, NA = not applicable

Comparison of chemotherapy response rates

FOLFIRINOX response at the different evaluation time points and final ORR and DCR are presented in Table 2. In total, 28 patients (LAPC, *n*=10, 7.4%; metastatic disease, *n*=18, 12.2%) stopped treatment due to unacceptable toxicity of FOLFIRINOX before CT evaluation was performed. At the first CT evaluation, 23.6% of metastatic patients and 10.3% of LAPC patients showed progressive disease (*P*=0.002), as determined by RECIST 1.1 criteria. The second CT evaluation, after eight cycles of FOLFIRINOX, revealed no statistically significant difference in treatment response between patient groups; 5.4% of metastatic patients and 5.1% of LAPC patients showed progressive disease (*P*=0.061). After twelve cycles of FOLFIRINOX, metastatic patients showed again more often progressive disease (8.1% vs 0%, *P*<0.001).

Table 2. FOLFIRINOX response outcomes in patients with locally advanced (LAPC) and metastatic pancreatic cancer, based on the RECIST 1.1 criteria.

	LAPC *n*=136 (%)	**Metastatic disease *n*=148 (%)**	***P***
CT evaluation 1[a]			
CR	0 (0)	0 (0)	0.002
PR	11 (8.1)	16 (10.8)	
SD	101 (74.3)	79 (53.4)	
PD	14 (10.3)	35 (23.6)	
Unknown	10 (7.4)	18 (12.2)	
CT evaluation 2[a]			
CR	0 (0)	0 (0)	0.061
PR	18 (13.2)	29 (19.6)	
SD	60 (44.1)	43 (29.1)	
PD	7 (5.1)	8 (5.4)	
Unknown	41 (30.2)	51 (34.5)	
NA	10 (7.4)	17 (11.5)	
Final response outcome[b]			
CR	0 (0)	0 (0)	<0.001
PR	19 (14.0)	27 (18.2)	
SD	86 (63.2)	49 (33.1)	
PD	21 (15.4)	53 (35.8)	
Unknown	10 (7.4)	19 (12.8)	
ORR	19/136 (14.0)	27/148 (18.2)	0.329
DCR	105/136 (77.2)	76/148 (51.4)	<0.001

CR = complete response, DCR = disease control rate, NA = not applicable, ORR = objective response rate, PD = progressive disease, PR = partial response, RECIST = response evaluation criteria in solid tumours, SD = stable disease.

[a] CT scan evaluations were performed after every fourth cycle of FOLFIRINOX, or earlier in case patients showed clinical signs of tumor progression or treatable disease symptoms that could be diagnosed with radiology. [b] Final response outcome was defined as the RECIST 1.1 treatment response measured on the latest available evaluation CT scan.

In the majority of patients in the LAPC group, the final response outcome was stable disease (63.2%), and in an additional 14% of patients the final response outcome was partial response. In LAPC, 15.4% of patients showed progressive disease during or immediately after FOLFIRINOX chemotherapy. In metastatic disease patients, 33.1% showed stable disease, 18.2% partial response, and 35.8% progressive disease ($P<0.001$). None of the patients showed a complete response. The ORR was similar between LAPC (14.0%) and metastatic disease patients (18.2%, $P=0.329$), but the DCR was significantly higher in LAPC patients (77.2% vs 51.4%, $P<0.001$).

The impact of disease stage and treatment response on survival

The median OS was 12.7 months (95% CI 11.4-14.1 months) in LAPC patients and 8.1 months (95% CI 6.5-9.6 months) in patients with metastatic PDAC ($P<0.001$). Kaplan-Meier curves are shown in Figure 1. The median PFS was longer in LAPC patients (10.0 months, 95% CI 8.5-11.5 months) versus metastatic disease patients (6.7 months, 95% CI 6.0-7.4 months, $P<0.001$). LAPC patients with partial response showed significantly longer median OS (23.0 months, 95% CI 17.3-28.7 months vs 15.8 months, 95% CI 14.6-17.0 months, $P=0.024$), though not significantly longer median PFS (12.0 months, 95% CI 8.5-15.5 months vs 10.4 months, 95% CI 6.0-14.8 months, $P=0.221$) compared to metastatic patients with partial response to FOLFIRINOX treatment. When comparing disease control patients, the LAPC patient group again was associated with better median OS (15.3 months, 95% CI 12.0-18.7 months vs 12.6 months, 95% CI 10.4-14.8 months, $P=0.040$) and median PFS (12.4 months, 95% CI 10.7-14.1 months vs 9.1 months, 95% CI 7.8-10.4 months, $P=0.004$) compared to metastatic disease patients. However, for patients with progressive disease during FOLFIRINOX chemotherapy, both median OS (7.7 months, 95% CI 3.5-11.8 months vs 5.5 months, 95% CI 4.5-6.6 months, $P=0.096$) and median PFS (2.3 months, 95% CI 1.8-2.8 months vs 2.3 months, 95% CI 1.6-2.9 months, $P=0.723$) did not significantly differ between LAPC and metastatic disease patients.

Conditional survival analysis

Conditional survival curves are presented in Figure 2. In LAPC patients, the probability of surviving two years increased from 14% at the start of FOLFIRINOX to 16%, 26%, and 42% with every additional six months survived up to 1.5 years. In metastatic patients, the probability of achieving 2-year survival increased from 10% to 15%, 29%, and 57%. The probability to survive one additional year, or 1-year CS, was 54% in LAPC patients at the start of FOLFIRINOX, then decreased to 26% after one year and increased to 79% after two years. In metastatic patients, the 1-year CS was 33%, then decreased to 29% after one year, and was 30% after two years.

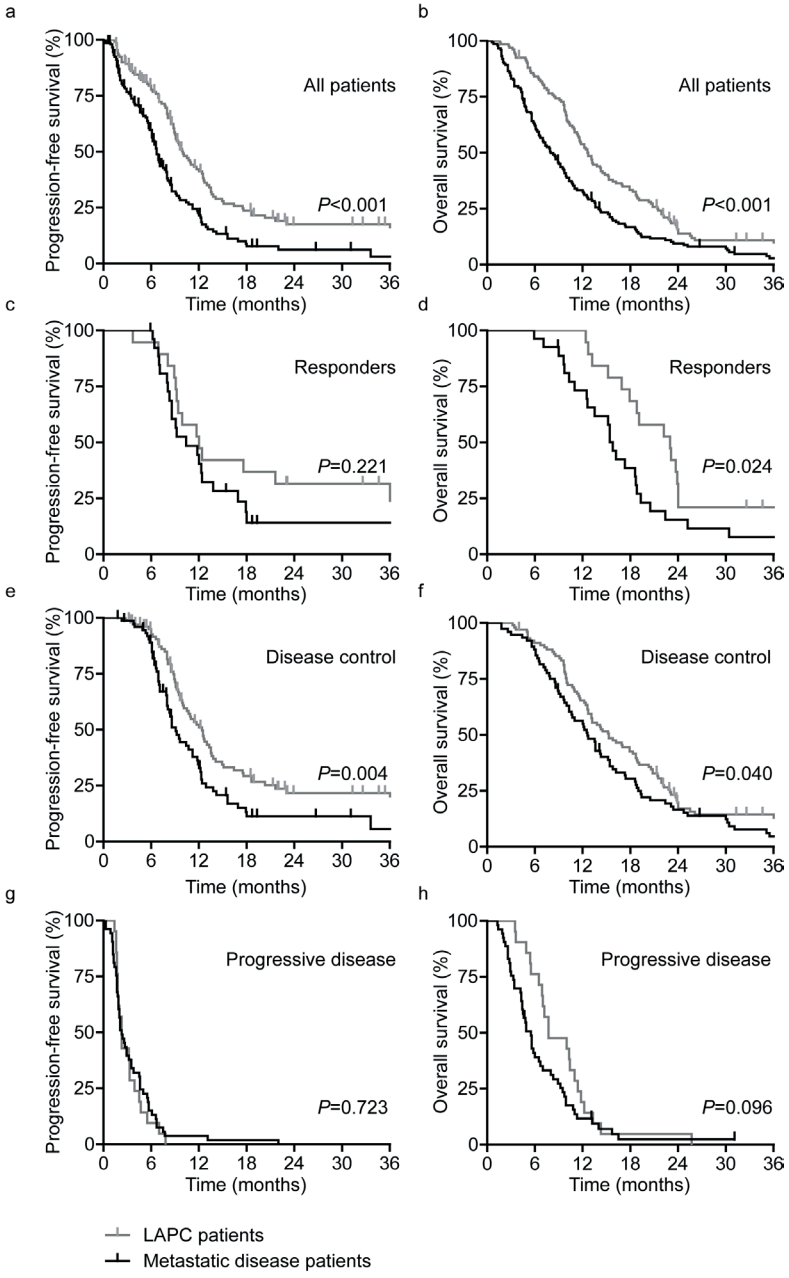
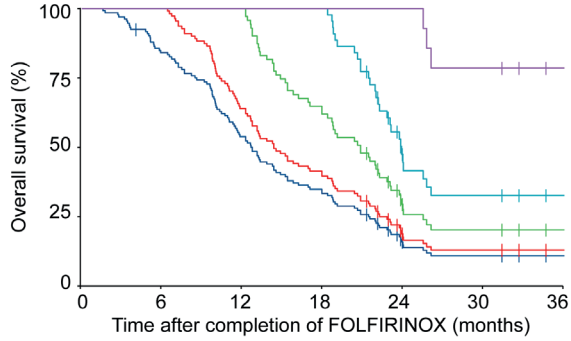


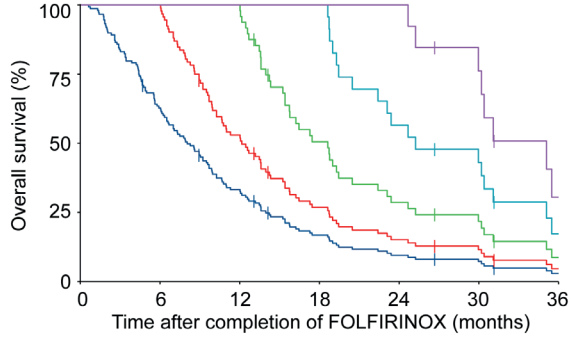
Figure 1. Kaplan-Meier estimates for progression-free survival and overall survival of patients with locally advanced pancreatic cancer (LAPC) and metastatic pancreatic cancer after treatment with FOLFIRINOX.
(a+b) Total cohorts of LAPC (*n*=136) and metastatic disease patients (*n*=148). (c+d) LAPC (*n*=19) versus metastatic disease patients (*n*=27) with partial response after FOLFIRINOX treatment, according to the RECIST 1.1 criteria. (e+f) LAPC (*n*=105) versus metastatic disease patients (*n*=76) with disease control after FOLFIRINOX, including partial response or stable disease. (g+h) LAPC (*n*=21) versus metastatic disease patients (*n*=53) with progressive disease during or immediately after FOLFIRINOX.

A



	Survival probability to reach x months						
Given conditional survival	0	6	12	18	24	30	36
0	100%	84%	54%	33%	14%	11%	11%
6		100%	64%	40%	16%	13%	13%
12			100%	62%	26%	20%	20%
18				100%	42%	33%	33%
24					100%	79%	79%
30						100%	100%
Number at risk	133	111	71	44	14	11	8

B



	Survival probability to reach x months						
Given conditional survival	0	6	12	18	24	30	36
0	100%	63%	33%	17%	10%	7%	3%
6		100%	53%	27%	15%	12%	5%
12			100%	51%	29%	22%	9%
18				100%	57%	43%	17%
24					100%	76%	30%
30						100%	40%
Number at risk	148	92	48	23	13	9	3

Figure 2. Kaplan-Meier estimates for conditional survival up to three years, given a survival of 6-30 months after completion of FOLFIRINOX in locally advanced pancreatic cancer (LAPC) patients (A) and metastatic disease patients (B).

DISCUSSION

In this retrospective cohort study, we evaluated differences in FOLFIRINOX response outcome and survival after FOLFIRINOX in patients with either LAPC or metastatic PDAC. We found similar ORR in the two patient groups, but significantly higher DCR in LAPC patients (77.2%) compared to patients with metastatic PDAC (51.4%). As expected, the median OS and PFS in LAPC patients were better than in patients with metastatic PDAC. However, in patients with progressive disease during or immediately after FOLFIRINOX, stage of disease did not influence survival. The response rate in advanced PDAC patients is satisfying and these outcomes might encourage physicians and patients to start with FOLFIRINOX treatment., especially because the majority of patients with advanced PDAC do not receive any palliative chemotherapy at this moment.[11] Also, LAPC patients report good quality of life after eight cycles of FOLFIRINOX, measured with validated questionnaires (F. van der Sijde *et al*., 2021, submitted).

This is the first study on CS after chemotherapy in advanced PDAC patients. Previous studies with CS analyses included patients with resectable disease,[6, 10, 12] which is the patient group with overall the highest survival rate. Real-world data from advanced PDAC patients undergoing chemotherapy treatment is, unfortunately, often lacking or hard to obtain. Therefore, patients are currently being informed about chemotherapy outcome based on information from clinical trials, often performed in highly selected patient cohorts. The benefit of treatment might be overestimated due to exclusion of patients with poor prognosis in clinical trials. Survival of patients included in our unselected cohort is far shorter than the reported median survival in literature. For example, a meta-analysis of 13 clinical trials describing FOLFIRINOX in LAPC patients reported a median OS of more than two years,[13] while our results show a median OS of 12.7 months in patients with similar stage of disease. From multiple clinical trials, the median OS in metastatic PDAC patients has been estimated at 10-11 months.[4] In this retrospective study, however, we found a median OS of 8 months with FOLFIRINOX. Furthermore, in the Netherlands, second-line chemotherapy or experimental treatment in clinical trials in case of progression are not often offered, which might partly explain these poor results.

In addition to OS, we consider CS an important outcome to be discussed with patients scheduled for FOLFIRINOX. Conditional survival analyses in this cohort showed that in both patient groups the survival probability increased after completion of FOLFIRINOX. Reassessment of survival probability after FOLFIRINOX might benefit patients; informing them about life expectancy can help patients in decision-making on future treatment or supportive care.

This study has some limitations. First, the retrospective design of the study might have biased the outcome. Chemotherapy schedules are standardized, though treating physicians might deviate from this at their own discretion, for example when patients are responding very well to treatment. In this cohort, some patients received more than eight or twelve cycles of FOLFIRINOX for LAPC or metastatic disease, respectively. In addition, patients could have stopped treatment at any time for any reason, not necessarily due to progression of disease only. Second, three out of four hospitals participating in this study, of which two academic hospitals, were relatively high volume centers. A previous study has shown that high hospital volume is associated with improved survival of PDAC patients treated with systemic therapy.[14] Third, CS is probably also influenced by other factors besides survival time. Increasing survival rates given CS indicate that patients with poor prognostic factors have already died early after FOLFIRINOX and only patients with beneficial tumor characteristics remain. In contrast to studies with CS analyses in patients with resectable PDAC, we do not have any information on tumor characteristics that might be associated with aggressive biological behavior,[10] such as tumor differentiation, invasiveness, and positive lymph nodes. Fourth, the sample sizes, and especially the low number of patients at risk in CS analyses, resulted in wide confidence intervals, particularly for outcomes longer after FOLFIRINOX.

CONCLUSION

This study describes outcomes of response rate, survival, and conditional survival of patients with locally advanced and metastatic pancreatic cancer treated with FOLFIRINOX. The disease control rate was higher and survival longer in LAPC patients compared to patients with metastastic disease. Overall survival was shorter in this real-world cohort compared to outcomes reported from clinical trials. One year after the start of chemotherapy, the probability to survive one more year is 15% to 30% in patients with advanced PDAC. These clinical results are useful to optimally counsel patients and could help in shared decision-making before start of chemotherapy and during follow-up.

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Chapter 3

Locally advanced pancreatic cancer patients report excellent quality of life after treatment with FOLFIRINOX

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ABSTRACT

Background Quality of life in cancer patients might be affected by chemotherapy-induced toxicity. Especially in patients with pancreatic ductal adenocarcinoma (PDAC), with a short life expectancy, fear of poor quality of life is often a reason for both patients and medical oncologists to refrain from further treatment. In this study, we investigated quality of life (QoL), pain, sleep, and activity levels in locally advanced pancreatic cancer (LAPC) patients after FOLFIRINOX treatment.

Methods A total of 41 LAPC patients with stable disease or partial response were included after completion of at least eight cycles of FOLFIRINOX. QoL was measured with the EORTC QLQ-C30 and NRS pain scores. Patients completed the Richard Campbell Sleep Questionnaire (RCSQ) for five consecutive nights and wore a GENEActiv tri-axial accelerometer (Actiwatch) for seven days, registering sleep duration, efficiency, and activity.

Results Mean EORTC QLQ-C30 score for global health status was 78.3 (± standard deviation 17.3), higher than reference values for cancer patients (P<0.001) and general population (P=0.045). LAPC patients reported few disease-related symptoms. Two patients (5%) reported pain scores >3. Mean sleep duration was 8 hours/night (±1.2 hours) and sleep efficiency 70% (±9%) with high patient-reported quality of sleep (mean RCSQ score 72.0±11.4). Mean duration of moderate-vigorous activity was 37 minutes/week (±103 minutes/week).

Conclusions QoL is excellent in LAPC patients with disease control after FOLFIRINOX, measured with validated questionnaires and Actiwatch registration. The expectation that QoL after FOLFIRINOX is poor, is not substantiated by this study and should not be a reason to refrain from treatment.

INTRODUCTION

FOLFIRINOX chemotherapy, a combination of fluorouracil, leucovorin, irinotecan and oxaliplatin, is standard-of-care for locally advanced pancreatic cancer (LAPC) patients with a good performance status. The median overall survival (OS) of these patients is 24.2 months with FOLFIRINOX.[1] The survival benefit of FOLFIRINOX is greater compared to other chemotherapy regimens, such as gemcitabine.[1, 2] Unfortunately, the response rate of FOLFIRINOX is rather low; approximately 10-30% of patients shows significant shrinkage of the primary tumor.[3, 4] However, in the majority of patients FOLFIRINOX will stabilize the disease and prevent early metastasis.[4] Nevertheless, medical oncologists are still cautious to administer FOLFIRINOX to pancreatic ductal adenocarcinoma (PDAC) patients because of the high toxicity rate.[5, 6] More than half of patients will experience FOLFIRINOX-related toxicity, including nausea and vomiting, diarrhea, fatigue, neuropathy, mucositis, thrombocytopenia, and neutropenia.[1, 4, 6] These toxicity-related symptoms might impact the quality of life of these patients and even be a reason to choose or switch to another, less toxic chemotherapy regimen, or stop treatment completely. Quality of life is especially important for patients with a short life expectancy, such as LAPC patients. Whether or not diminished quality of life is worth the survival benefit is of course a very personal decision that should be made by patients themselves, but it is the treating physician that should properly inform them on the pros and cons of (chemotherapy) treatment. Next to chemotherapy-induced toxicity symptoms, patients can of course also suffer from disease associated symptoms. Depending on the tumor location, patients often report duodenal obstruction, icterus, and exocrine pancreatic insufficiency,[7] which might be diminished by the administration of chemotherapy. In addition, it is known that many PDAC patients show signs of sleep problems, anxiety, depression, and pain.[7, 8] However, if patients still report these symptoms, and what their quality of life is after chemotherapy, has not been studied yet.

For that reason, the aim of this article was to investigate the quality of life of LAPC patients after treatment with FOLFIRINOX, based on validated questionnaires for quality of life and sleep, Actiwatch activity and sleep registration, and patient-reported pain scores. Additionally, quality of life, activity, sleep, and pain were compared between patients with short (<12 months) and long (>12 months) survival after completion of FOLFIRINOX treatment.

MATERIALS AND METHODS

Patient selection

LAPC patients were selected from a single-center, prospective clinical trial (Dutch trial register NL7578) investigating the safety and efficacy of adding IMM-101 immunotherapy, a suspension of heat-killed whole cell mycobacterium obuense, to the treatment for LAPC patients with FOLFIRINOX followed by stereotactic body radiation therapy (SBRT). Patients were included in the study after completion of at least four cycles of FOLFIRINOX and before the start of SBRT combined with IMM-101 between October 2019 and January 2021. Other inclusion criteria were age 18-75 years, world health organization (WHO) performance status <2, and American Society of Anesthesiologists (ASA) classification <III. Patients were excluded if they showed progressive disease during or immediately after FOLFIRINOX, since they were not eligible for radiation therapy anymore. Patients were also excluded when they had received previous chemotherapy other than FOLFIRINOX, if they were or had been treated with immunotherapeutic drugs or immunosuppressive drugs. Also, patients with immunodeficiency, a history of Human Immunodeficiency Virus (HIV) infection, or active hepatitis B or C were excluded. This trial, and the side study on quality of life, was approved by the medical ethics review board of the Erasmus Medical Center Rotterdam (MEC-2019-0219). All patients provided written informed consent and the study was conducted in accordance with the declaration of Helsinki.

Study procedure

Upon inclusion in the study, after completion of FOLFIRINOX and before start of SBRT with IMM-101, patients started wearing a GENEActiv tri-axial accelerometer (Actiwatch) to register their activity and sleep for seven days and filled out the Dutch language version of the Richard Campbell Sleep Questionnaire (RCSQ) for five consecutive days. In addition, they filled out the Dutch language version of the EORTC QLQ-C30 quality of life questionnaire. Patients reported pain scores using a numeric rating scale (NRS) of 0-10 at time of inclusion as part of routine care.

Measurement instruments

The European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire-C30 (EORTC QLQ-C30) is a validated 30-item questionnaire of self-reported health-related quality of life of cancer patients containing both single- and multi-item measures, including global health status/overall quality of life, five functional scales (physical, role, cognitive, emotional, and social functioning), three symptom scales (fatigue, pain, and nausea/vomiting), and six single items (constipation, diarrhea, insomnia, dyspnea, appetite loss, and financial difficulties). Higher scores for global health status

and functional scales suggest better quality of life and functioning, while higher scores for symptoms represent more symptoms and thus worse quality of life.[9] An overview of items in the EORTC QLQ-C30 is presented in Supplementary Table 1.

The validated RCSQ contains five aspects of sleep: sleep depth, falling asleep (sleep latency), number of awakenings, returning to sleep, and overall quality of sleep. Each item is scored on a visual analogue scale (VAS) of 0–100. Higher scores represent better sleep quality. Scores between 0 and 25 represent very poor sleep, scores of 26-50 poor sleep, scores 51-75 good sleep, and 76-100 very good sleep.[10-12] The items of the RCSQ are shown in Supplementary Table 2.

The GENEActiv tri-axial accelerometer (Activinsights, Kimbolton, UK) is a wrist-worn accelerometer that provides raw movement data, light, temperature, and posture change measurements. It measures bed time, rise time, elapsed sleep time, sleep time, sleep efficiency, activity levels and the amount of time of moderate to vigorous activity.[13] Sleep efficiency is the percentage of sleep time out of the total time between bed time and rise time. The World Health Organization (WHO) recommends that adults should do at least 150 minutes of moderate intensity or 75 minutes of vigorous intensity activity throughout a week.[14]

Patient characteristics, such as age and sex, and FOLFIRINOX chemotherapy specifics, such as start date and number of cycles received, medication use, and follow-up data were retrieved from medical records by a medical doctor.

Statistical analysis

EORTC QLQ-C30 questionnaire scores were compared to reference values for cancer patients (*n*=23,553), stage III-IV cancer patients (*n*=8,066), liver/bile/pancreas cancer patients (*n*=750), and general population (*n*=7,802) with a summary data two sample t-test. Reference values were taken from the online dataset of the EORTC Quality of Life Group.[15] The accelerometer raw data files were downloaded and processed with R-package GGIR, version 2.3-0 (http://cran.r-project.org). Repeated measurements of, for example, sleep duration and time spent on activities, were averaged per day or night. Correlation between RCSQ questionnaires and EORTC quality of life was tested with Pearson's correlation coefficient. Questionnaire and Actiwatch results were compared between patients with short (<12 months) and patients with long (>12 months) overall survival (OS), calculated from the last day of FOLFIRINOX, with independent samples t-tests. Data were analyzed with SPSS Statistics version 25 (IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

In total, 41 LAPC patients were included in this study with a median age of 63 years (range 41-76 years). Table 1 presents the patient characteristics. All patients had received at least eight cycles of FOLFIRINOX before inclusion. After FOLFIRINOX, twelve patients (29.3%) showed partial response of the tumor, the other 29 patients (70.7%) stable disease. The median time between the last cycle of FOLFIRINOX and filling out the questionnaires and start of Actiwatch registration was 28 days (range 5-96 days). At the time of analysis, after a median follow-up of 7.9 months, 26 patients (63.4%) had progressive disease, and 14 patients (34.2%) had died.

Table 1. Patient characteristics.

	LAPC cohort (*n*=41)
Age (years), median (range)	63 (41-76)
Sex, male (%)	18 (43.9)
WHO performance status (%)	
0	12 (29.3)
1	29 (70.7)
Number of cycles of FOLFIRINOX received (%)	
8	35 (85.4)
9	4 (9.8)
12	2 (4.9)
RECIST[a] tumor response (%)	
Stable disease	29 (70.7)
Partial response	12 (29.3)
Time between last cycle of FOLFIRINOX and questionnaires (days), median (range)	28 (5-96)
Use of pain medication at time of inclusion, yes (%)	16 (39.0)
Type of pain medication	
Paracetamol	5 (12.2)
Opioids	3 (7.3)
Neuropathic pain medication[b]	1 (2.4)
Paracetamol + NSAIDs	2 (4.9)
Paracetamol + opioids	4 (9.8)
Opioids + neuropathic pain medication	1 (2.4)
EORTC QLQ-C30 questionnaire available (%)	40 (97.6)
Richard Campbell Sleep Questionnaire available (%)	38 (92.7)
Actiwatch registration (%)	36 (87.8)

EORTC QLQ-C30 = European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, LAPC = locally advanced pancreatic cancer, NSAIDs = nonsteroidal anti-inflammatory drugs. [a] According to the RECIST 1.1 criteria for CT scan evaluations. [b] For example gabapentin, or amitriptyline.

Quality of life after FOLFIRINOX treatment

EORTC QLQ-C30 questionnaires were available for 40 patients. The reported answers per questionnaire item can be found in Table 2. The mean score for global health status in this cohort was 78.3 (± standard deviation 17.3). This score was significantly higher than the reported reference values for cancer patients (61.3±24.2, $P<0.001$), stage III-IV cancer patients (61.5±23.6, $P<0.001$), liver/bile/pancreas cancer patients (55.9±25.1, $P<0.001$)

Table 2. Single item answers to the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC QLQ-C30) for the LAPC cohort (*n*=40).

Item	Category	Not at all, *n* (%)	A little, *n* (%)	Quite a bit, *n* (%)	Very much, *n* (%)
Strenuous activities	PF	12 (30.0)	18 (45.0)	9 (22.5)	0 (0)
Long walk	PF	9 (22.5)	21 (52.5)	8 (20.0)	1 (2.5)
Short walk	PF	30 (75.0)	8 (20.0)	2 (5.0)	0 (0)
Bed or chair	PF	29 (72.5)	11 (27.5)	0 (0)	0 (0)
Self care	PF	37 (92.5)	3 (7.5)	0 (0)	0 (0)
Limited in work	RF	14 (35.0)	18 (45.0)	6 (15.0)	2 (5.0)
Limited in leisure	RF	21 (52.5)	13 (32.5)	3 (7.5)	3 (7.5)
Dyspnea	DY	26 (65.0)	8 (20.0)	4 (10.0)	2 (5.0)
Pain	PA	24 (60.0)	10 (25.0)	4 (10.0)	2 (5.0)
Need to rest	FA	10 (25.0)	20 (50.0)	9 (22.5)	1 (2.5)
Insomnia	SL	22 (55.0)	14 (35.0)	3 (7.5)	1 (2.5)
Felt weak	FA	12 (30.0)	21 (52.5)	4 (10.0)	3 (7.5)
Appetite loss	AP	25 (62.5)	12 (30.0)	2 (5.0)	1 (2.5)
Nausea	NV	33 (82.5)	7 (17.5)	0 (0)	0 (0)
Vomiting	NV	38 (95.0)	1 (2.5)	1 (2.5)	0 (0)
Constipation	CO	31 (77.5)	7 (17.5)	0 (0)	1 (2.5)
Diarrhea	DI	26 (65.0)	11 (27.5)	3 (7.5)	0 (0)
Felt tired	FA	9 (22.5)	23 (57.5)	8 (20.0)	0 (0)
Pain interference	PA	34 (85..0)	3 (7.5)	2 (5.0)	1 (2.5)
Concentration	CF	25 (62.5)	12 (30.0)	2 (5.0)	0 (0)
Tension	EF	23 (57.5)	15 (37.5)	2 (5.0)	0 (0)
Worry	EF	16 (40.0)	18 (45.0)	6 (15.0)	0 (0)
Irritability	EF	24 (60.0)	15 (37.5)	0 (0)	1 (2.5)
Depression	EF	29 (72.5)	10 (25.0)	1 (2.5)	0 (0)
Memory trouble	CF	23 (57.5)	15 (37.5)	2 (5.0)	0 (0)
Family life	SF	26 (65.0)	10 (25.0)	1 (2.5)	3 (7.5)
Social activities	SF	20 (50.0)	13 (32.5)	3 (7.5)	4 (10.0)
Financial difficulties	FD	36 (90.0)	3 (7.5)	0 (0)	1 (2.5)

AP = appetite loss, CF = cognitive functioning, CO = constipation, DI = diarrhea, DY = dyspnea, EF = emotional functioning, FA = fatigue, FD = financial difficulties, LAPC = locally advanced pancreatic cancer, NV = nausea and vomiting, PA = pain, PF = physical functioning, RF = role functioning, SF = social functioning, SL = insomnia.

and general population (71.2±22.4, *P*=0.045), as presented in Figure 1a. In Supplementary Table 3, all EORTC QLQ-C30 scores for our LAPC cohort and reference values are shown.

Patients in this LAPC cohort scored higher on the emotional functioning scale (83.6±16.0) compared to all reference cohorts (*P*<0.001 for cancer cohorts, *P*=0.043 for general population), shown in Figure 1b. LAPC patients also scored higher on the physical functioning scale (83.2±12.4) compared the stage III-IV cancer (*P*=0.003) and liver/bile/pancreas cancer (*P*=0.025) reference cohorts. On physical functioning (83.2±12.4 vs 89.8±16.2, *P*=0.010), role functioning (73.3±27.1 vs 84.7±25.4, *P*=0.005), and social functioning (78.4±29.2 vs 87.5±22.9, *P*=0.012), LAPC patients scored lower than the general population reference cohort.

In Figure 1c reported symptom scores are presented. Compared to the liver/bile/pancreas cancer reference cohort, our LAPC cohort scored lower on the symptom scales for nausea/vomiting (4.2±10.5 vs 14.2±22.5, *P*=0.005), pain (14.2±23.1 vs 29.6±32.8, *P*=0.003), insomnia (19.1±24.9 vs 32.2±34.4, *P*=0.016), appetite loss (15.8±23.8 vs 32.3±37.2, *P*=0.005), constipation (8.5±19.7 vs 20.4±31.3, *P*=0.016), and financial difficulties (5.0±17.7 vs 21.9±32.5, *P*=0.001). Compared to the general population, LAPC patients reported a higher score for fatigue (32.7±21.2 vs 24.1±24.0, *P*=0.024), appetite loss (15.8±23.8 vs 6.7±18.3, *P*=0.002), and diarrhea (14.1±21.2 vs 7.0±18.0, *P*=0.013).

Pain after FOLFIRINOX treatment

In accordance with the EORTC QLQ-C30 outcome, the LAPC patients in this cohort did not often report symptoms of pain, measured with NRS. Only two patients reported an NRS score of >3: 1 patient NRS 4, 1 patient NRS 7. The patient with NRS 4 immediately started opioid treatment after inclusion. The patient with NRS 7 showed very early progression of disease, within two months after inclusion. Four of the patients reporting any symptoms of pain did not use any pain medication at the time of measurement.

Sleep quantity and quality after FOLFIRINOX treatment

Objective outcome of sleep, measured with the Actiwatch, was available from 36 patients. The mean sleep duration was 8.0 hours/night (±1.2 hours/night), based on a registration period of seven consecutive nights. The mean sleep efficiency was 69.6% (±9.0%).

RCSQ questionnaires were available from 38 patients. The questionnaires were filled out during the first five consecutive nights of Actiwatch registration. The mean RCSQ score calculated from all five items during five nights was 72.0 (±11.4). The scores per item per

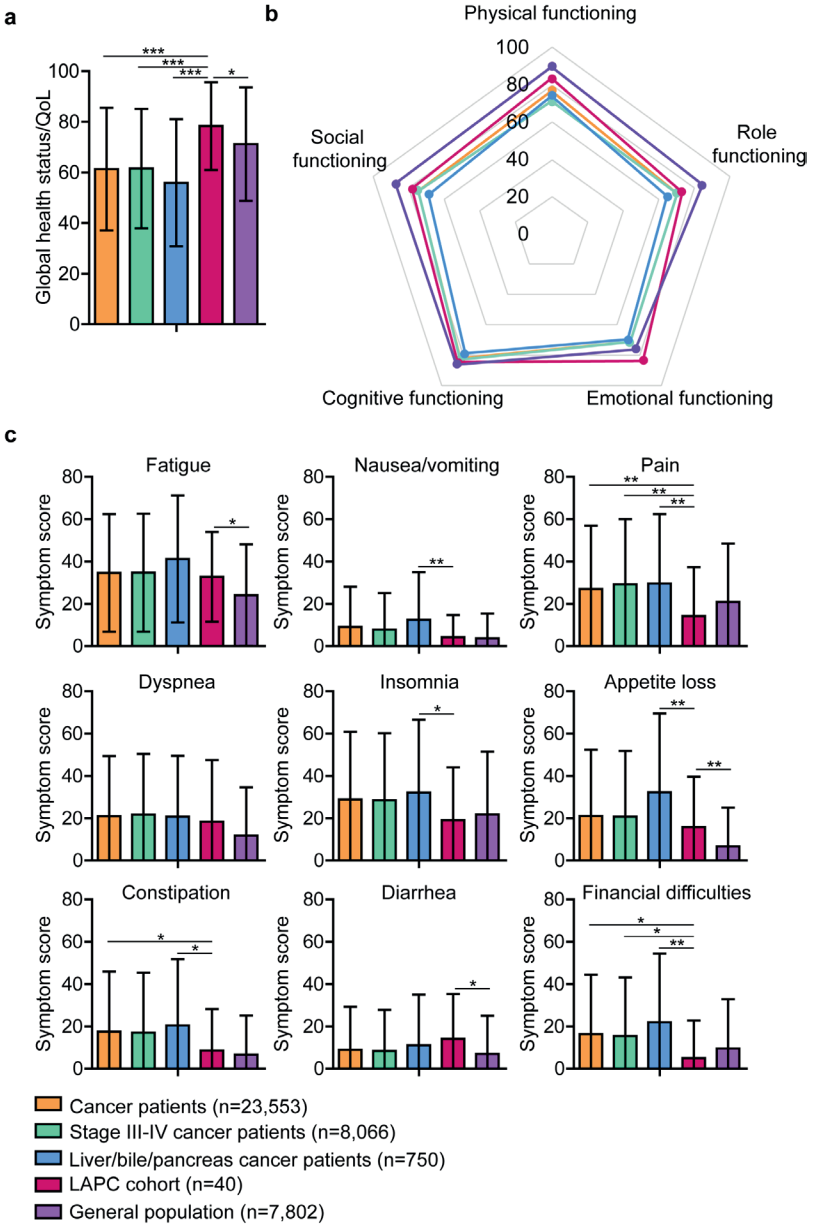

Figure 1. European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ-C30) outcomes for the locally advanced pancreatic cancer (LAPC) cohort compared to the reference values for cancer patients and general population.

a. Comparison of global health status or quality of life (QoL) between the LAPC cohort and reference cancer patient groups and general population. b. Scores of functional scales for the LAPC cohort and reference cancer patient groups and general population. c. Comparison of symptom scores between the LAPC cohort and reference cancer patient groups and general population.

Higher scores for global health status and functional scales suggest better quality of life and functioning, higher scores for symptoms represent more symptoms. *P<0.05, **P<0.01, ***P<0.001, calculated with t-tests.

night are shown in Table 3. Patients reported the lowest scores for sleep depth (mean score 66.2±18.7), and the highest scores for returning to sleep after being awaken (mean score 77.5±11.9).

Table 3. Single item answers to the Richards-Campbell Sleep Questionnaire (RCSQ) for the LAPC cohort (*n*=38).

Item	Mean score (SD) night 1	Mean score (SD) night 2	Mean score (SD) night 3	Mean score (SD) night 4	Mean score (SD) night 5	Mean score (SD) total period
Sleep depth	64.3 (23.0)	63.1 (26.0)	65.0 (23.6)	69.1 (21.0)	69.2 (22.6)	66.2 (18.7)
Sleep latency	69.1 (23.7)	72.4 (20.2)	75.5 (20.1)	71.1 (24.9)	74.9 (20.6)	72.5 (14.4)
Awakenings	73.3 (20.6)	72.2 (18.3)	73.0 (17.6)	74.2 (17.5)	74.0 (20.7)	73.6 (13.4)
Returning to sleep	77.1 (18.4)	79.3 (13.7)	76.8 (16.9)	77.4 (15.6)	75.2 (20.3)	77.5 (11.9)
Sleep quality	73.8 (21.2)	78.2 (16.3)	75.2 (20.2)	77.5 (17.4)	73.3 (24.1)	75.5 (13.0)
Total score	70.9 (15.3)	71.9 (13.4)	72.5 (16.4)	73.0 (15.9)	72.3 (19.2)	72.0 (11.4)

LAPC = locally advanced pancreatic cancer, SD = standard deviation.

There was not a significant correlation between patient-reported RCSQ scores and global health status/quality of life (Pearson r=0.18; 95% confidence interval (CI) -0.17-0.48, *P*=0.306), as presented in Supplementary Figure 1.

Activity level after FOLFIRINOX treatment

Objective activity registration, measured with the Actiwatch, was available for 32 patients. Only 11/32 patients (34.4%) registered a period of moderate to vigorous activity at one or multiple days. The mean duration of moderate or vigorous activity was 5.3 minutes/day (±14.8 minutes/day), based on a registration period of seven consecutive days. When only including patients with at least one moderate-vigorous activity registered, the mean duration of moderate-vigorous activity was 37 minutes/week (±103 minutes/week). Only three patients (9.4%) did more than 75 minutes of moderate-vigorous activity during the week, as recommended by the WHO.

Quality of life in patients with short overall survival after FOLFIRINOX

In Table 4, the most important EORTC QLQ-C30, RCSQ and Actiwatch results are shown for patients with an overall survival of at least twelve months (*n*=11) and patients that died within twelve months (*n*=11) after completion of FOLFIRINOX. There were no differences between groups in patient-reported quality of life, based on the EORTC QLQ-C30 global health status item (mean score 80.4±13.9 for long survival, 76.5±21.5 for short survival patients, *P*=0.619). There was a difference in patient-reported fatigue (*P*=0.024): patients with a survival longer than twelve months reported more fatigue symptoms (mean score 45.4±22.7), while patients with a short survival reported lower fatigue symptoms (24.1±17.8). In both groups, 3/11 (27.3%) of patients reported a pain score of

NRS >0. Sleep efficiency, but not sleep duration or sleep quality, was better in patients with OS >12 months (76.1±5.0%) compared to patients with OS <12 months (68.3±9.8%, *P*=0.039).

Table 4. Comparison of EORTC QLQ-C30, RCSQ, and Actiwatch results between patients with long and short overall survival (OS) after completion of FOLFIRINOX.

	Patients with OS >12 months (*n*=11), mean score (SD)	**Patients with OS <12 months (*n*=11), mean score (SD)**	***P***
Global health status (QoL)	80.4 (13.9)	76.5 (21.5)	0.619
Physical functioning	81.2 (15.4)	85.0 (11.1)	0.520
Role functioning	71.2 (30.8)	72.6 (34.4)	0.918
Emotional functioning	82.6 (15.1)	90.8 (13.3)	0.193
Cognitive functioning	80.3 (91.7)	91.7 (14.1)	0.161
Social functioning	63.6 (33.1)	80.5 (26.5)	0.204
Fatigue	45.4 (22.7)	24.1 (17.8)	0.024
Nausea and vomiting	4.6 (10.7)	0.0 (0.0)	0.175
Pain	15.3 (23.0)	21.2 (33.4)	0.634
Dyspnea	24.2 (33.7)	21.2 (34.3)	0.838
Insomnia	30.2 (31.5)	9.1 (21.6)	0.082
Appetite loss	24.2 (33.7)	9.0 (15.4)	0.189
Constipation	12.1 (30.8)	6.0 (13.4)	0.554
Diarrhea	15.1 (22.9)	12.0 (16.7)	0.721
Financial difficulties	12.1 (30.8)	6.0 (13.4)	0.554
Pain score NRS>0, yes (%)	3 (27.3)	3 (27.3)	1.000
Sleep duration, hours	8.6 (0.9)	7.8 (1.4)	0.142
Sleep efficiency (%)	76.1 (5.0)	68.3 (9.8)	0.039
Sleep quality (RCSQ)	72.4 (15.2)	74.0 (10.7)	0.783

EORTC QLQ-C30 = European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, QoL = quality of life, RCSQ = Richard Campbell Sleep Questionnaire, SD = standard deviation.

DISCUSSION

In this cohort study, we investigated the quality of life of LAPC patients after completion of FOLFIRINOX chemotherapy. We found that patients within our LAPC cohort reported high quality of life scores and low symptom scores, measured with the EORTC QLQ-C30 quality of life questionnaire. Quality of life scores were better than the reported scores

for cancer reference cohorts and even better than general population references. LAPC patients reported more symptoms of fatigue, appetite loss, and diarrhea compared to the general population, but less frequent than cancer reference cohorts, including PDAC patients. The majority of LAPC patients (73%) reported no pain symptoms, with or without the use of pain medication. Patients also showed sufficient sleep duration (8 hours/night) and sleep efficiency (70%), objectively measured by Actiwatch registration, and they reported high quality of sleep. The activity level of LAPC patients was, however, very low. A minority of patients in this cohort did some moderate to vigorous activity during the registration period. Only three patients reached the activity level recommended by the WHO. The activity level of this LAPC cohort is lower compared to previous published data on activity levels in patients with different types of cancer (e.g. lymphoma, breast cancer, head and neck cancer, colon cancer).[16]

Not many studies have published on quality of life in PDAC patients during or after treatment. A systematic review on the incidence and overall burden of PDAC in Europe showed, based on data from three different cohorts, that PDAC patients report worse quality of life compared to the general population.[17] This data, however, was retrieved at time of diagnosis from patients of all disease stages, before start of any treatment and is, therefore, not necessarily comparable to our data. Studies that investigated the change in quality of life after treatment, measured with the EORTC QLQ-C30, all showed better results after treatment compared to baseline measurements. Patients with resectable disease showed improving quality of life results after operation, despite extensive surgical procedures.[18, 19] Also in metastatic disease patients, included in a phase II trial to investigate the response to and toxicity of FOLFIRINOX, quality of life improved after treatment.[20] One study showed that patients eligible for (chemotherapy) treatment reported better quality of life compared to those who only received best supportive care.[21] Also, patients treated with palliative care were more satisfied with the given care than patients treated with curative intent.[18]

Our results are in line with the literature. We hypothesize that PDAC patients in this cohort, despite their poor prognosis, reported good to excellent quality of life for a couple of reasons. First, patients in this study all received treatment and toxicity-related symptoms of FOLFIRINOX might pass by quickly after treatment is completed. Second, in patients responding to treatment, FOLFIRINOX will diminish the disease load and by that the symptom burden.[22-24] This is affirmed by the results of other quality of life studies in PDAC patients after treatment.[18-21] Third, undergoing treatment might also have psychological benefit. Any treatment, standard chemotherapy or experimental immunotherapy for example, will give hope for curation or improved survival. Instead of waiting for disease progression, these patients have done everything they can to improve their

life expectancy. Also, previous research has shown that patients participating in clinical trials receive better quality of care resulting in better patient outcome.[25, 26] Finally, these patients answered the questionnaires several months after diagnosis. By that time, they might have come to peace with the disease and its prognosis. Because of these positive results, we believe that treating physicians should maybe be less reluctant in considering FOLFIRINOX as treatment in LAPC patients.

A limitation of this study is that we could not include all patient who have started FOLFIRINOX. However, in our previous cohort study, only 12% of patients with LAPC showed progressive disease during FOLFIRINOX treatment, the vast majority had stable disease,[27] and the effectiveness of FOLFIRINOX as first-line treatment for LAPC patients has been published.[1, 3]

This is the first study on quality of life in LAPC patients treated with FOLFIRINOX and the results can be used to help inform patients during shared decision-making. However, our data is based on a relatively small sample size (n=41) with no comparison to baseline data or data at time of diagnosis. Also, patient outcome might be positively biased because all patients included in this clinical trial showed at least stable disease after FOLFIRINOX treatment. It would be interesting to investigate the quality of life of patients with progressive disease during treatment in the future.

CONCLUSION

LAPC patients with disease control after FOLFIRINOX treatment report excellent quality of life, very little symptoms and pain, and good sleep quality and sleep duration after treatment with FOLFIRINOX. The expectation of poor quality of life after FOLFIRINOX should not be a reason for treating physicians to refrain from treatment.

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SUPPLEMENTARY FILES

Supplementary Table 1. European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ-C30), English language version 3.

Item	Not at all	A little	Quite a bit	Very much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a long walk?	1	2	3	4
3. Do you have any trouble taking a short walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4
During the past week:				
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your family life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your social activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4
For the following questions please circle the number between 1 and 7 that best applies to you				
29. How would you rate your overall health during the past week?	1	2	3	4 5 6 7
30. How would you rate your overall quality of life during the past week?	1	2	3	4 5 6 7

Supplementary Table 2. Richard Campbell Sleep Questionnaire (RCSQ), English language version.

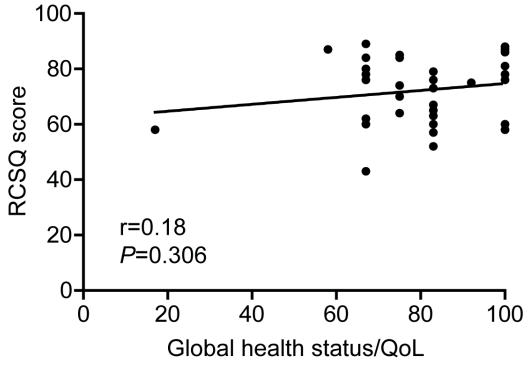
Item	Question	Answer*	
Sleep depth	My sleep last night was:	Light sleep (0)	Deep sleep (100)
Sleep latency	Last night, the first time I got to sleep, I:	Just never could fall asleep (0)	Fell asleep almost immediately (100)
Awakenings	Last night, I was:	Awake all night long (0)	Awake very little (100)
Returning to sleep	Last night, when I woke up or was awakened, I:	Couldn't get back to sleep (0)	Got back to sleep immediately (100)
Sleep quality	I would describe my sleep last night as:	A bad night's sleep (0)	A good night's sleep (100)

*Each question is scored on a 100-mm visual analogue scale.

Supplementary Table 3. Scores of the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ-C30) for the LAPC cohort compared to the reference values for cancer patients and general population.

	LAPC cohort (*n*=40), mean score (SD)	Cancer patients (*n*=23,553), mean score (SD)	*P*	Stage III-IV cancer patients (*n*=8,066), mean score (SD)	*P*	Liver/bile/ pancreas cancer patients (*n*=750), mean score (SD)	*P*	General population (*n*=7,802), mean score (SD)	*P*
Global health status (QoL)	78.3 (17.3)	61.3 (24.2)	<0.001	61.5 (23.6)	<0.001	55.9 (25.1)	<0.001	71.2 (22.4)	0.045
Physical functioning	83.2 (12.4)	76.7 (23.2)	0.076	71.2 (25.8)	0.003	74.1 (25.7)	0.025	89.8 (16.2)	0.010
Role functioning	73.3 (27.1)	70.5 (32.8)	0.589	70.6 (32.8)	0.603	65.2 (36.3)	0.159	84.7 (25.4)	0.005
Emotional functioning	83.6 (16.0)	71.4 (24.2)	0.001	71.5 (23.8)	0.001	69.8 (25.5)	0.001	76.3 (22.8)	0.043
Cognitive functioning	85.0 (17.0)	82.6 (21.9)	0.488	83.2 (21.3)	0.594	79.0 (23.1)	0.101	86.1 (20.0)	0.728
Social functioning	78.4 (29.2)	75.0 (29.1)	0.460	75.1 (28.9)	0.471	69.0 (31.7)	0.061	87.5 (22.9)	0.012
Fatigue	32.7 (21.2)	34.6 (27.8)	0.666	34.7 (27.9)	0.651	41.2 (30.0)	0.074	24.1 (24.0)	0.024
Nausea and vomiting	4.2 (10.5)	9.1 (19.0)	0.103	7.8 (17.3)	0.189	14.2 (22.5)	0.005	3.7 (11.7)	0.787
Pain	14.2 (23.1)	27.0 (29.9)	0.007	29.2 (30.8)	0.002	29.6 (32.8)	0.003	20.9 (27.6)	0.125
Dyspnea	18.3 (29.2)	21.0 (28.4)	0.548	21.7 (28.7)	0.455	20.8 (28.7)	0.583	11.8 (22.8)	0.073
Insomnia	19.1 (24.9)	28.9 (31.9)	0.052	28.5 (31.7)	0.061	32.2 (34.4)	0.016	21.8 (29.7)	0.566
Appetite loss	15.8 (23.8)	21.1 (31.3)	0.284	20.8 (31.0)	0.308	32.3 (37.2)	0.005	6.7 (18.3)	0.002
Constipation	8.5 (19.7)	17.5 (28.4)	0.045	17.0 (28.4)	0.059	20.4 (31.3)	0.016	6.7 (18.4)	0.537
Diarrhea	14.1 (21.2)	9.0 (20.3)	0.112	8.3 (19.5)	0.061	11.1 (23.9)	0.428	7.0 (18.0)	0.013
Financial difficulties	5.0 (17.7)	16.3 (28.1)	0.011	15.4 (27.7)	0.018	21.9 (32.5)	0.001	9.5 (23.3)	0.223

LAPC = locally advanced pancreatic cancer, QoL = quality of life, SD = standard deviation.



Supplementary Figure 1. Correlation plot of Richard Campbell Sleep Questionnaire (RCSQ) scores and global health score/quality of life as measured with the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ-C30) questionnaire. There was no significant correlation (*P*=0.306) between patient-reported sleep quality and patient-reported quality of life (Pearson's r=0.18; 95% confidence interval (CI) -0.17-0.48).

Part II

Genetic markers for prediction of treatment response and prognosis

Chapter 4

Circulating biomarkers for prediction of objective response to chemotherapy in pancreatic cancer patients

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ABSTRACT

Pancreatic cancer is a lethal disease with increasing incidence. Most patients present with advanced disease, for which palliative systemic chemotherapy is the only therapeutic option. Despite improved median survival rates with FOLFIRINOX or gemcitabine chemotherapy compared to best supportive care, many individual patients may not benefit from chemotherapy. Biomarkers are needed to predict who will benefit from chemotherapy and to monitor patient's response to chemotherapy. This review summarizes current research and future perspectives on circulating biomarkers for systemic chemotherapy response.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the twelfth most common cancer worldwide. Its incidence is rising; PDAC will likely be the second leading cause of cancer-related deaths in 2030, exceeding the mortality from colorectal and breast cancer.[1] The mortality rate of PDAC is almost equal to the incidence rate, with a 5-year survival rate of only 7.7%.[2] Approximately 80% of patients are diagnosed with advanced disease and are therefore not eligible for tumor resection.[3] For those patients chemotherapy is currently the standard treatment while more effective therapies are lacking. Patients with resectable PDAC are increasingly offered neoadjuvant treatment instead of upfront surgery followed by adjuvant systemic treatment. Preoperative chemotherapy is likely to be the future standard.[4-11]

Different chemotherapy regimens are offered, but FOLFIRINOX (a combination of fluorouracil, leucovorin, irinotecan and oxaliplatin) is currently the treatment of choice for patients with metastatic disease and locally advanced pancreatic cancer (LAPC). For metastatic PDAC, FOLFIRINOX is superior to gemcitabine in terms of patient survival benefit.[12-14] The median overall survival (OS) of metastatic PDAC patients is 11.1 months when treated with FOLFIRINOX, compared to 6.8 months when treated with gemcitabine.[13] A recent meta-analysis has shown that this holds as well for LAPC. The median OS in patients treated with FOLFIRINOX was 24.2 months[14] versus 6-13 months in patients treated with gemcitabine.[15, 16] Because of its lower toxicity profile, gemcitabine is still often used, particularly in patients with a poor performance status[13] and as adjuvant treatment after surgical resection. Only few articles have been published about adjuvant FOLFIRINOX treatment. A recent randomized controlled trial showed a median OS of 54.4 months in the FOLFIRINOX group versus 34.8 months in the gemcitabine group.[17] It should be mentioned, that adjuvant gemcitabine combined with capecitabine might be more effective than gemcitabine monotherapy. The ESPAC-4 trial showed a median overall survival of 28.0 months for gemcitabine plus capecitabine versus 25.5 months for gemcitabine monotherapy.[18]

In a multicenter, randomized, phase 2–3 trial in metastatic patients, FOLFIRINOX treatment was found associated with higher response rates than gemcitabine, based on the RECIST criteria for radiologic monitoring of the disease.[13, 19] Nevertheless, a recent meta-analysis still showed a response rate of only 25-30% in metastatic patients.[20] This could mean that the large majority of patients will suffer from side effects without benefiting from FOLFIRINOX treatment. Toxicity is a serious problem in chemotherapy treatment. Especially with the FOLFIRINOX combination regimen, grade 3 and 4 toxicity rates are as high as 60-70%.[13, 14, 20-22] For gemcitabine monotherapy, toxicity rates are significantly

lower.[13, 23, 24] Identifying non-responders is crucial to avoid exposing patients to the adverse events and incurring costs of ineffective treatment.

Currently, response to chemotherapy is evaluated with the use of computed tomography (CT) scans. The RECIST criteria classify response into four classes. Complete response (CR) is defined as disappearance of all target lesions; partial response (PR) as a decrease of >30% in the sum of diameters of target lesions; and progressive disease (PD) is defined as an increase of >20% in the sum of diameters of target lesions or the appearance of a new cancer lesion. Stable disease (SD) shows neither sufficient shrinkage nor increase to be classified as PR or PD.[19] In case of (partial) response or SD, treatment will be continued, while PD is a reason for treatment discontinuation. Although the RECIST criteria have been validated for multiple solid cancers and show a good inter- and intra-observer agreement,[25-27] the value of radiologic response assessment for PDAC is still being debated. Measurements of the primary tumor are difficult and less reliable in view of its invasive growth pattern and fibrotic aspects combined with inflammation.[28, 29] Moreover, treatment response is not only reflected by a decrease in tumor size. Therefore, predictive biomarkers for chemotherapy response, and for FOLFIRINOX in particular, in PDAC patients would be of great value to spare patients this toxic treatment.

A biomarker is a measurable characteristic that can be used as an indicator of physiologic and pathologic processes. Two main categories are distinguished: prognostic and predictive biomarkers. Prognostic biomarkers provide insight into patient outcome, independent of the received treatment. These biomarkers can give an estimation of survival, time to disease progression, or type of progression. Predictive biomarkers, on the other hand, can identify subsets of patients who would benefit from specific cancer treatment, and thus can guide treatment decisions. A biomarker can only be termed predictive if its predictive value was confirmed in a study with at least two treatment comparison groups, preferably a randomized controlled trial.[30] An ideal biomarker is cheap to obtain, easy to analyze, non-invasive for the patient, and can be collected repeatedly.

Circulating biomarkers are gaining interest in cancer research due to the many advantages over tumor tissue biomarkers. For PDAC as well as other cancers, liquid (blood) biopsies are preferred in the search of predictive and prognostic biomarkers, because the procedure is less hazardous than percutaneous or endoscopic tumor biopsies. Not all PDAC patients undergo a tumor biopsy and the primary tumor is resected in only a minority of patients. Moreover, the representation of intratumoral heterogeneity in a single tumor biopsy is limited, which limitation can possibly be overcome by using liquid biopsies. These represent a more complete make-up of the primary tumor and/or metastatic sites.[31] Blood samples, e.g. plasma or serum, are a great source of cell-free

DNA, RNA, exosomes, proteins and metabolites. Repeated measurements in peripheral blood are easy to obtain and preferred over multiple tumor biopsies.[32]

In this review, we will discuss current research and future perspectives on circulating biomarkers predictive for chemotherapy response used for pancreatic cancer patient stratification.

MATERIALS AND METHODS

In August 2018, Embase, PubMed, Medline, Web of Science, Cochrane Library, and Google Scholar were searched using the search terms "pancreatic neoplasms", "biomarkers", "genetic markers", "chemotherapy", "chemoradiotherapy", "treatment outcome", and "treatment response" without year restrictions.

Eligible studies were those using liquid biopsies to investigate predictive biomarkers for response in PDAC patients. Studies that only investigated biomarkers in tissue or did not report the source of biomarkers were excluded, as well as prognostic biomarker studies and biomarker studies on chronic pancreatitis or neuroendocrine tumors of the pancreas. Studies had to report objective response to chemotherapy according to the RECIST criteria. This review article focuses on FOLFIRINOX and gemcitabine treatment, because the majority of PDAC patients receive either one of these therapies. For that reason, only studies that included at least one patient treated with FOLFIRINOX or gemcitabine chemotherapy were included. Letters, editorials, expert opinions, case reports, and non-English language studies were excluded. Eligible studies and those for which information in the abstract was not sufficient for exclusion were read in full. Bibliographies of included publications were searched for other studies.

RESULTS

A total of 27 articles met the inclusion criteria and will be discussed in this review.

Conventional tumor markers

Carbohydrate antigen 19-9 (CA19-9) is a sialylated Lewis blood group antigen associated with different cancers including PDAC.[33] This marker was first described by Koprowski et al. as a tumor associated antigen in colorectal cancer cell lines.[34] CA19-9 is widely studied as a diagnostic, prognostic and predictive biomarker in PDAC. However, up to 20% of the population is Lewis negative and cannot synthesize CA19-9.[35]

Thirteen articles deal with the role of conventional tumor markers as a predictor of chemotherapy response. For an overview, see Table 1. Four of the studies concerned focus on the predictive value of baseline levels of CA19-9 to predict objective response. Posttreatment CA19-9 levels were measured in two studies. Twelve studies investigated the predictive value of changes in CA19-9 levels over time.

Table 1. Overview of studies including conventional tumor markers as predictive biomarkers for chemotherapy response in pancreatic cancer patients.

Author	Year of publication	Number of patients	Stage of disease	Treatment	Biomarker
Gogas [40]	1998	39	LAPC (*n*=21) + metastatic (*n*=18)	5-FU + cisplatin + epirubicine	CA19-9
Halm [41]	2000	36	LAPC + metastatic	Gemcitabine	CA19-9
Stemmler [53]	2003	77	LAPC + metastatic	Gemcitabine + cisplatin	CA19-9
Ali [42]	2007	18	LAPC + metastatic	Gemcitabine	CA19-9
Wong [44]	2008	75	Metastatic	Gemcitabine + cisplatin (*n*=41) Gemcitabine + cisplatin + bevacizumab (*n*=34)	CA19-9
An [38]	2009	61	LAPC + metastatic	Gemcitabine Gemcitabine + oxaliplatin Gemcitabine + 5FU/CF	CA19-9
Koom [37]	2009	69	Borderline resectable + LAPC	Gemcitabine + radiation Paclitaxel + radiation	CA19-9
Yoo [39]	2011	84	LAPC	Radiation + 5-FU (*n*=53) Radiation + capecitabine (*n*=31)	CA19-9
Tsutsumi [46]	2012	90	LAPC + metastatic	Gemcitabine	CA19-9 SPAN-1
Boeck [36]	2013	68	LAPC + metastatic + recurrence	Gemcitabine Gemcitabine + erlotinib Gemcitabine + everolimus Gemcitabine + axitinib Gemcitabine + WX-671 Capecitabine Capecitabine + erlotinib Nab-paclitaxel	CA19-9 CEA
Azzariti [47]	2016	27	Metastatic	FOLFIRINOX (*n*=21) Gemcitabine + nab-paclitaxel (*n*=6)	CA19-9
Chiorean [45]	2016	454	Metastatic	Gemcitabine (*n*=202) Gemcitabine + nab-paclitaxel (*n*=252)	CA19-9
Robert [48]	2017	160	Metastatic	FOLFIRINOX (*n*=85) Gemcitabine (*n*=75)	CA19-9

LAPC = locally advanced pancreatic cancer, CA19-9 = carbohydrate antigen 19-9, CEA = carcinoembryonic antigen.

Baseline CA19-9

Boeck *et al.* investigated baseline CA19-9 levels in 68 patients with LAPC, metastatic, and recurrent disease before receiving gemcitabine- or capecitabine-based treatment. In patients with non-progressive disease (SD+PR+CR), the median baseline level was 341.5 U/mL. In patients with PD after chemotherapy, the median baseline level was 5810.5 U/mL (P=0.006).[36] Koom *et al.* measured CA19-9 levels before chemoradiotherapy (gemcitabine or paclitaxel) in 69 patients with borderline resectable PDAC or LAPC. The median level in responders (PR+CR) was 661 U/mL; that in non-responders (PD+SD) was 518 U/mL (P=0.78).[37] An *et al.* reported a median CA19-9 level of 682 U/mL in the complete study population, including 61 patients with LAPC and metastatic disease receiving gemcitabine-based treatment. The median CA19-9 level was 682 U/mL. The objective response rate (ORR) in the patients with a baseline level below 682 U/mL was 43.5% versus 15.8% in the patients with a baseline level above 682 U/mL (P=0.051).[38] In article of Yoo *et al.,* 84 LAPC patients underwent chemoradiotherapy with 5-FU or capecitabine. The response rate, including CR and PR, in patients with a baseline CA19-9 level below 100 U/mL was 51.9% versus 37.5% in patients with a baseline CA19-9 level between 101-400 U/mL and 15.2% in patients with a baseline CA19-9 above 400 U/mL (P=0.009).[39] In conclusion, baseline CA19-9 levels in non-responders are higher than those in responders.

Posttreatment CA19-9

Boeck *et al.* and Koom *et al.* investigated the role of posttreatment CA19-9 levels as a predictor of radiologic response. Boeck *et al.* found different posttreatment levels in patients with PD and patients with non-PD. At the time of re-staging, eight weeks after start of treatment, median CA19-9 levels were 135.0 U/mL in patients with non-PD and 6428.0 U/mL in patients with PD (P<0.001).[36] In contrast to baseline CA19-9 levels, posttreatment CA19-9 levels did statistically significant differ between responders and non-responders. Koom *et al.* reported a median CA19-9 level in responders of 80 U/mL versus 199 U/mL in non-responders (P=0.001).[37] This means that not only baseline levels, but also posttreatment levels of CA19-9 are higher in patients without response to treatment.

CA19-9 changes

Several authors looked into the predictive value of decreasing CA19-9 levels under chemotherapy. Boeck *et al.* quantified changes in CA19-9 from baseline at different time points. After eight weeks, for example, median CA19-9 levels in patients with non-PD had decreased by 65.2% versus 17.4% in patients with PD (P<0.001).[36] In the study by Koom *et al.,* the median decrease from baseline in responders was 93% and in non-responders 72% (P=0.002).[37] In the study by An *et al.* the decrease in patients

with CA19-9 level ≥25% was 47.8% versus 10.5% in the group with <25% decrease.[38] Gogas *et al.* studied 39 patients with LAPC and metastatic disease, all treated with a combination of 5-FU, cisplatin and epirubicin. A decrease in CA19-9 ≥15% was considered biochemical response to treatment; an increase ≥15% was considered biochemical progression of disease. CA19-9 decrease showed a sensitivity of 67%, specificity of 69%, positive predictive value (PPV) of 20% and negative predictive value (NPV) of 87% for partial response as based on radiologic findings. CA19-9 increase seemed a slightly better prediction tool for progression with a sensitivity of 86%, specificity of 67%, PPV of 37% and NPV of 90%.[40] Halm and colleagues defined CA19-9 response as a decrease of >20% and quantified CA19-9 levels in 36 patients with LAPC and metastatic disease, treated with gemcitabine. After eight weeks of treatment, four patients achieved partial objective response, as measured on CT scans. All four of these patients showed a CA19-9 decrease >20%, indicating biochemical response. Still, nineteen out of 25 patients with SD showed the same biochemical response to treatment, but also two out of seven patients with PD.[41] It is hard to draw a conclusion because *P*-values are not provided. Ali *et al.* also defined CA19-9 response as a decrease of ≥20% and studied eighteen LAPC and metastatic patients who received gemcitabine treatment. Seven patients out of nine with stable disease and only one patient with partial response on CT scan showed a CA19-9 decrease ≥20%. Two out of eight patients with progressive disease showed decrease in CA19-9 over time.[42] *P*-values are not provided. Stemmler *et al.* chose a cut-off point of 50% decrease for biochemical response to treatment and studied 77 patients with LAPC and metastatic disease, treated with gemcitabine and cisplatin. All of the complete responders on CT imaging showed also biochemical response. Seventy of the patients with partial response (91%) were biochemical responders. Fifty-four of the patients with progressive disease on CT (70%) did not have a CA19-9 response as defined above. This resulted in a sensitivity of 93.3%, specificity of 53.2%, PPV of 32.5% and NPV of 97.1%.[43] Wong *et al.* only included patients with metastatic disease. All 75 patients were treated with gemcitabine-based treatment. The cut-off point for biochemical response to treatment was set at 75% decrease. All (13/13) patients with objective response, 15/43 patients with stable disease and only 1/19 patients with progressive disease showed >75% decrease in CA19-9 levels during treatment ($P<0.0001$).[44] The largest study investigating tumor marker CA19-9 as a predictor of response is the one by Chiorean *et al.,* who included 454 patients diagnosed with metastatic disease and treated with gemcitabine with or without nab-paclitaxel. In this study, the ORR was low. Forty of the 252 patients treated with gemcitabine+nab-paclitaxel and 13/202 patients treated with gemcitabine alone showed response to treatment (CR+PR). In the combination treatment group, 38/40 responders showed any decrease in CA19-9 after 8 weeks of treatment. All 13 responders in the monotherapy group also showed any decrease. Hundred and fifty-eight of the 199 patients with SD (79%) in the nab-paclitaxel group had decreasing CA19-9

levels over time versus 133/170 (78%) in the gemcitabine group. No specific results were given for patients with progressive disease.[45] Tsutsumi *et al.* studied 90 patients with LAPC and metastatic disease, all treated with gemcitabine chemotherapy. CA19-9 levels after one month of treatment were compared to baseline levels. Patients with SD showed a decrease of 12%; patients with partial response a decrease of 68%; and patients with PD showed a median increase of 27% in CA19-9 level.[46] The study by Azzariti *et al.* is the only study investigating CA19-9 in metastatic patients treated with FOLFIRINOX. In total, 27 patients were included, of whom 21 received FOLFIRINOX and six gemcitabine. Pretreatment and posttreatment levels of CA19-9 were compared within PR, SD and PD patient groups. In the PR group, the mean pretreatment CA19-9 level was 831.5 U/mL and the mean posttreatment level 355.75 U/mL ($P<0.01$). The corresponding figures for the SD group were 521.4 U/mL and 292.7 U/mL (not significant) and for the PD group 2569.67 U/mL and 3384.44 U/mL ($P<0.05$).[47] The authors did not provide inter-group comparisons, but a pattern is shown of decreasing CA19-9 levels over time in patients with response and decreasing CA19-9 levels over time in patients with progression. The most recent CA19-9 study was conducted by Robert *et al.* in 2017 as part of the ACCORD11/PRODIGE4 trial, comparing FOLFIRINOX to gemcitabine chemotherapy in 160 metastatic patients. For the patient group receiving FOLFIRINOX, the ORR at 8 weeks of treatment was 44.0% in patients with a CA19-9 decrease of ≥20% versus 22.9% in patients with a decrease of <20%. For the patient group receiving gemcitabine, the ORR was 23.1% in patients with ≥20% decrease and 0% in patients with a <20% decrease.[48] Although the above studies used different definitions of CA19-9 increase and decrease, the overall consensus is that CA19-9 levels decrease over time in responders and do not decrease or even increase in non-responders.

CEA

Only one study, by Boeck *et al.,* focused on carcinoembryonic antigen (CEA), another broadly used tumor marker in cancer prognostics.[49, 50] Median CEA levels at baseline were statistically significant different between responders and non-responders. Baseline levels were 3.7 ng/mL in patients with SD or PR and 17.1 ng/mL in patients with PD ($P=0.008$). Measurement after 8 weeks showed median CEA levels of 2.6 ng/mL in patients with SD or PR and 18.1 ng/mL in patients with PD ($P=0.002$). Patients with response to treatment had a median decrease of CEA of 26.3%, while patients with PD showed no decrease of CEA levels at all ($P=0.078$).[36]

SPAN-1

Tsutsumi *et al.* did not only measure the tumor marker CA19-9, but also SPAN-1, a high molecular weight glycoprotein. SPAN-1 is expressed by many pancreatic cancers.[51] This tumor marker has already been investigated as a diagnostic and prognostic tool for

PDAC. It is little used however, because its sensitivity and specificity are no higher than those of existing tumor markers such as CA19-9.[51, 52] In the study by Tsutsumi *et al.,* a SPAN-1 change pattern was found similar to that of CA19-9. The median SPAN-1 level decreased by 24% and 48%, respectively, in patients with SD and PR and increased by 11% in patients with PD.[46]

Genetic markers

Single nucleotide polymorphisms

The term genetic markers encompass germline single nucleotide polymorphisms (SNPs), somatic cancer mutations, as well as differences in RNA expression. SNPs are variations of a single nucleotide in the DNA and may be associated with development of disease and response to treatment.[53] Theoretically these can all serve as predictors of tumor growth, treatment response or metastasis.[32] An overview of articles describing genetic markers for prediction of chemotherapy is given in Table 2. Two published studies have investigated several SNPs for response to chemotherapy. One, by Dong *et al.,* included 131 resectable patients treated with neoadjuvant gemcitabine based chemotherapy. It focuses on fifteen SNPs in genes already known for their roles in DNA mismatch repair (MMR).[54] MMR is necessary for recognition, removal and repair of DNA damage generated during DNA replication. Deficiencies in MMR could interfere with the response to chemotherapy, because chemotherapeutic stress on the tumor cells results in decreased apoptosis.[55] Five of the 15 SNPs were associated with response to preoperative chemotherapy. Genotype *MSH2* G322D with SNP GG was associated with an ORR of 88.1%; genotype *MSH2* G322D with SNP GA/AA was associated with an ORR of 63.2% (P=0.04). *MSH2* IVS12-6T>C with SNP TT was associated with an ORR of 92.8%, versus 71.9% with SNP TC/CC (P<0.001). Genotype *MSH3* P231P with SNP GG was associated with an ORR of 89.2%, versus 65.4% with the GA/AA genotype (P=0.002). ORR for *TREX1* Ex14-460C>T with SNP TT/CT and CC were 88.5% and 75.6% respectively (P=0.047). *TP73* Ex2+4G>A with SNP GG showed an ORR of 94.3%, SNP GA/AA 72.2% (P<0.001).[54] The second study, by Tanaka *et al.,* selected 17 SNPs that have been found involved in de metabolism of gemcitabine. 149 LAPC patients were included who all received gemcitabine-based chemotherapy and radiation. Only two genotypes were associated with objective response to treatment. The ORR for genotype *CDA* A-76C (K27Q) with SNP AA was 72.4% and for SNP AC/CC 51.8% (P=0.017). The ORR for *hENT1* A-201G with SNP AA/AG was 95.0% and for SNP GG only 33.3% (P=0.019).[56] Obviously, comparison of these two articles is futile because they address different SNPs. Together, however, they make clear that even small genetic alterations can influence a patient's response to chemotherapy.

Table 2. Overview of studies including genetic markers as predictive biomarkers for chemotherapy response in pancreatic cancer patients.

Author	Year of publication	Number of patients	Stage of disease	Treatment	Biomarker
Dong [54]	2009	131	Resectable	Gemcitabine + radiation Gemcitabine + cisplatin + radiation	SNPs (*MSH2* G322D, *MSH2* IVS12-6T>C, *MSH3* P231P, *TREX1* Ex14-460C>T, *TP73* Ex2+4G>A)
Tanaka [56]	2010	149	LAPC	Gemcitabine (+ radiation) Gemcitabine + cisplatin (+ radiation) Gemcitabine + oxaliplatin (+ radiation)	SNPs (*CDA* A-76C, *hENT1* A-201G)
Tjensvoll [63]	2016	14	LAPC (*n*=2) + metastatic (*n*=12)	Gemcitabine (*n*=6) FOLFIRINOX (*n*=8)	ctDNA (*KRAS*)
Cheng [60]	2017	13	Metastatic	Gemcitabine + nab-paclitaxel	ctDNA (*BRCA2, KRAS 12G, KRAS G12V, KRAS G12D, ERBB2, EGFR, KDR*)
Del Re [62]	2017	27	LAPC (*n*=4) + metastatic (*n*=23)	FOLFIRINOX (*n*=13) Gemcitabine + nab-paclitaxel (*n*=14)	ctDNA (*KRAS*)
Wang [66]	2017	62	LAPC (*n*=21) + metastatic (*n*=41)	Gemcitabine + cisplatin (*n*=24) Gemcitabine + nab-paclitaxel (*n*=14) Gemcitabine + oxaliplatin (*n*=24)	lncRNAs (*PVT1, HOTTIP, MALAT1*)
Perets [61]	2018	17	Metastatic	Unknown	ctDNA (*KRAS*)

LAPC = locally advanced pancreatic cancer, ctDNA = circulating tumor DNA, SNP = single nucleotide polymorphism.

Circulating tumor DNA

Circulating cell-free tumor DNA (ctDNA) is released in the blood stream as a result of apoptosis or necrosis of tumor cells and represents the molecular make-up of the cancer cells. Blood sampling is much less invasive than taking tumor biopsies and they can represent cancer heterogeneity to a larger extent compared to a single section from the primary tumor.[31] ctDNA blood levels are higher in patients with larger tumors and tumors that are well vascularized due to increased shedding into the circulation.[57] Theoretically, in case of chemotherapy response, therapy-induced tumor cell death should lead to an increase in ctDNA levels. In practice, however, ctDNA levels will then eventually become undetectable as eliminated cancer cells are no longer shedding their DNA. On the long term, increasing ctDNA levels could indicate disease progression as a result of increasing tumor load. The exact mechanism and timing of ctDNA shedding during and after anti-cancer treatment is not well understood. Moreover, the ctDNA dynamics may

be different for drugs with different mechanisms of action. On the other hand, ctDNA can also be used to investigate specific tumor mutations that could be associated with treatment response and prognosis. Most studies that analyzed mutant ctDNA levels were conducted in a prognostic setting. For example, the presence of ctDNA in plasma before treatment was found associated with worse OS in PDAC patients.[58, 59] Cheng *et al.* investigated the ctDNA mutation levels of different genes, including *KRAS*, which has been identified as one of the key players in pancreatic tumorigenesis. Mutational levels were measured at baseline and every eight weeks during chemotherapy with gemcitabine and nab-paclitaxel in thirteen metastatic patients. Serial plasma samples were monitored for several mutations, including *KRAS, BRCA2* and *EGFR*. Ten of these thirteen patients had detectable ctDNA mutations in various genes. While decreasing levels or undetectable levels of ctDNA in the blood over time were associated with objective response, increasing levels were associated with progressive disease. ctDNA levels even increased before progression was visible on CT scan. Of those ten patients, eight gained new ctDNA mutations during treatment.[60] Perets *et al.* investigated ctDNA patterns in seventeen patients with metastatic PDAC treated with chemotherapy, details of which were not provided. *KRAS* ctDNA levels during and after treatment increase over time in case of disease progression, as evaluated on radiological imaging. Also in this study, ctDNA levels tended to increase even before progression was visible on CT scans.[61] A study by Del Re *et al.* investigated ctDNA levels in thirteen patients receiving FOLFIRINOX as well as 14 patients receiving gemcitabine with nab-paclitaxel with LAPC or metastatic disease. A decrease in the amount of mutant *KRAS* ctDNA during or after the first cycle of chemotherapy showed a trend towards a better disease control rate (CR+PR+SD) as shown on the first radiologic evaluation scan. The difference in decrease of mutant *KRAS* ctDNA between responders and non-responders was not statistically significant (P=0.059).[62] In a pilot study by Tjensvoll *et al.* the level of mutant *KRAS* ctDNA prior to and during chemotherapy treatment was also significantly higher in patients that developed PD compared to those with SD. Here, too, ctDNA levels increased before disease progression was visible on radiologic imaging.[63] The fourteen patients with LAPC or metastatic disease in this study received either gemcitabine or FOLFIRINOX, but with regard to the ctDNA findings, the authors do not distinguish between the two treatments. In none of the aforementioned studies, standardized cut-off values for decrease or increase in ctDNA levels were determined. From these articles we can conclude that detectable or increasing ctDNA levels are associated with progressive disease. ctDNA analysis is a promising method for anti-cancer treatment monitoring - even though application in clinical practice is hindered by the lack of established reference values for ctDNA detection levels and fluctuations and the lack of measurement technique standardization.

Long non-coding RNAs

Long non-coding RNAs (lncRNA) are RNAs that do not code for proteins.[64] Still, they are important regulators of gene expression and thought to be associated with cancer, cancer recurrence or progression, metastasis, and prognosis.[65] In a recently published study by Wang *et al.,* several lncRNAs were investigated in peripheral blood samples from 62 patients with LAPC or metastatic disease, treated with gemcitabine based treatment. Baseline expression of lncRNAs, preselected based on their association with PDAC according to literature, was measured using real-time polymerase chain reaction (qPCR). The expression levels of three of the fourteen lncRNAs investigated showed promise for the prediction of response to treatment. The response rate (CR+PR) in patients with low expression of the lncRNA *PVT1* was 37.1% versus 14.8% in patients with a high expression ($P<0.001$). The corresponding figures for HOTTIP were 37.9% and 18.2% ($P<0.001$), and those for *MALAT1* 41.1% and 10.7% ($P=0.007$).[66] These three lncRNAs have previously been described as prognostic markers in pancreatic cancer. *HOTTIP* for example was found to be upregulated in PDAC tissues. *HOTTIP* silencing can result in proliferation arrest an decrease cell invasion. When *HOTTIP* is inhibited, antitumor effects of gemcitabine are enhanced.[65] *PVT1* has been identified as a regulator of gemcitabine sensitivity, in that overexpression of *PVT1* results in decreased sensitivity.[67] *MALAT1*, too, has been described as a poor prognostic factor in PDAC patients.[68] *MALAT1* promotes tumor proliferation and metastasis through activation of autophagy.[69]

Immunologic markers

Many cancers, including PDAC, may arise from chronic inflammation. Systemic inflammation is often observed in patients with cancer, as a result of the antitumoral response of the host in an attempt to induce tumor destruction. Systemic inflammation can also be tumor-induced, however, and in turn lead to neoplastic progression caused by tumor-promoting effects of immune cells.[70] See Table 3 for an overview of articles investigating predictive immunologic biomarkers for response.

Systemic inflammation ratios

Systemic inflammation ratios, such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR) and systemic immune inflammation index (SII), reflect the antitumor inflammation capacity of the host and are of prognostic value in PDAC patients.[71,72] One study, by Gao *et al.,* investigated the role of the NLR and PLR as a predictive marker in 122 LAPC and metastatic patients, most of whom received gemcitabine treatment. The mean baseline NLR level was 3.81, the mean PLR level was 142.14. Patients were separated in groups with a low NLR or PLR, or with a high NLR or PLR, using the mean values as cutoff. Disease control (PR+SD) after treatment was accomplished in 47/60 patients (78%) with low NLR levels and in 29/62 patients (47%) with high NLR levels ($P<0.001$). Disease control was accomplished in 33/60 patients (55%) with a low PLR and in 43/62 patients (69%) with a high PLR ($P=0.102$).[73]

Table 3. Overview of studies including immunologic markers as predictive biomarkers for chemotherapy response in pancreatic cancer patients.

Author	Year of publication	Number of patients	Stage of disease	Treatment	Biomarker
Palagani [82]	2012	4	Metastatic	5-FU + oxaliplatin + irinotecan and/ or avastin	CD44+ cells
Vizio [76]	2012	58	LAPC + metastatic	Gemcitabine (*n*=28) Gemcitabine + oxaliplatin (*n*=23) Bevacizumab + capecitabine + radiation (*n*=6) 5-FU + levofolinate calcium (*n*=1)	IL-23 IL-17a TGF-β1
Wittwer [86]	2013	68	LAPC + metastatic + recurrence	Gemcitabine Gemcitabine + erlotinib Gemcitabine + everolimus Gemcitabine + axitinib Capecitabine Capecitabine + erlotinib Nab-paclitaxel	sRAGE
Wittwer [87]	2013	68	LAPC (*n*=9) + metastatic (*n*=42) + recurrence (*n*=17)	Gemcitabine Capecitabine	sRAGE
Azzariti [47]	2016	27	Metastatic	FOLFIRINOX (*n*=21) Gemcitabine + nab-paclitaxel (*n*=6)	sCD40L
Gao [73]	2017	122	LAPC + metastatic	Gemcitabine (*n*=119) Fluorouracil (*n*=1) Capecitabine (*n*=1) S1 (*n*=1)	NLR
Usul Afsar [77]	2017	20	Metastatic	Gemcitabine (*n*=11) Gemcitabine + platinum (*n*=6) Gemcitabine + capecitabine (*n*=3)	IL-18

5-FU = fluorouracil, LAPC = locally advanced pancreatic cancer, IL = interleukin, sRAGE = soluble receptor for advanced glycation end products, NLR = neutrophil-to-lymphocyte ratio, IL = interleukin, S1 = tegafur, gimeracil and oteracil potassium.

Immune cells and cytokines

Regarding the working mechanism of subsets of immune cells instead of absolute numbers of immune cells, regulatory T cells (Tregs) are certainly of interest in immunologic biomarker research in PDAC patients. Tregs are essential in the regulation of immune responses, mostly to maintain tolerance to autoantigens. In cancer, however, Tregs can also suppress antitumor immune response, resulting in an ideal environment for tumor growth, and are associated with poor prognosis.[74, 75] Inhibitory cytokines produced by Tregs, such as TGF-β, interleukin-10 (IL-10) and interleukin-35 (IL-35), but also other immune inhibitory cytokines or proinflammatory cytokines produced by other cells and T cells have been studied as prognostic and predictive biomarkers.

Two studies have investigated different cytokines as a predictor of response to treatment. The study by Vizio *et al.* included 58 patients with LAPC or metastatic disease, mainly treated with gemcitabine. Pretreatment levels of interleukines (IL) IL-23, IL-17a and TGF-β1 were significantly lower in responders than in non-responders. The median pretreatment level of IL-23 was 4.68 pg/mL in responders versus 33.17 pg/mL in non-responders (P=0.030). The corresponding figures for IL-17a were 0 pg/mL versus 5.65 pg/mL (P=0.040) and 1469.8 pg/mL versus 1912.85 pg/mL for TGF-β1 (P=0.032).[76] Interleukin-18 (IL-18), a proinflammatory cytokine produced by macrophages, is also associated with treatment response, according to the study by Usul Afsar *et al.* In twenty patients with metastatic disease, treated with gemcitabine, lower pretreatment IL-18 levels were also associated with response. The median IL-18 level in responders (PR+SD) was 1273.8 pg/mL versus 1942.8 pg/mL in non-responders (P=0.04).[77]

Other interesting immunologic cells are CD44-positive cells. CD44 is a cell-surface glycoprotein involved in cell–cell and cell–matrix interactions and is known to be a cancer stem cell marker. It plays not only a role in cell adhesion, presentation of chemokines, and lymphocyte activation, but also in tumor development and metastasis.[78] Although CD44 is expressed in healthy tissue, it is often upregulated in different cancer types, including PDAC.[79-81] One published study, by Palagani *et al.,* has investigated the role of CD44 as a predictor for response to chemotherapy. Only four patients with metastatic PDAC were included, treated with a combination of 5-FU, oxaliplatin, irinotecan and/or avastin. The expression of CD44 almost immediately decreased after chemotherapy (P<0.05). The level of decrease was associated with objective response, with better outcomes for patients with lower CD44 expression after chemotherapy.[82]

Another cell-surface interaction molecule, CD40L, has been investigated as a predictor of response. CD40L is expressed by T cells and plays a role in the adaptive immune response.[83] The soluble form, sCD40L, is produced by activated T cells, but also by proinflammatory and prothrombotic platelets, leading to elevated serum levels in patients with cancer and autoimmune disorders.[84] Azzariti *et al.* investigated sCD40L levels in addition to CA19-9 levels, as described before in this review. In patients with PR, mean pretreatment values of sCD40L decreased over time from 11,718.05 pg/mL versus 4,689.42 pg/mL (P<0.01). In patients with PD, mean pretreatment values increased from 9,351.51 pg/mL to 22,282.92 pg/mL (P<0.01) after treatment. Serum sCD40L levels in patients with stable disease had not statistically significantly changed.[47]

The high molecular group box 1 protein (HMGB1) is a nuclear protein involved in DNA organizing and transcription. During necrosis or apoptosis, the HMGB1 protein is released from human cells into the blood stream. A massive release of HMGB1, induced

by chemotherapy, will stimulate the immune system such that an anti-tumor response is induced, as well as a pro-tumor effect at the long term.[85] The main receptor of HMGB1, the soluble receptor for advanced glycation end products (sRAGE), has a blocking effect on its extracellular pro-inflammatory functions by binding the protein. This regulation mechanism is thought to be a prognostic factor in cancer and treatment response.[85, 86] Wittwer *et al.* studied 68 patients treated with mainly gemcitabine and compared Levels of sRAGE between responders (SD+CR+PR) and non-responders before start of treatment, at days 21 and 42 of treatment, and after treatment at time of staging. Both pretreatment levels and day 21 levels were not significantly different between groups. At day 42, sRAGE levels were higher in responders than in non-responders: median 0.94 ng/mL versus 0.75 ng/mL, respectively (P=0.047).[87] This pattern was still sustained at day 56: median 1.09 ng/mL versus 0.79 ng/mL.[86]

DISCUSSION

PDAC has a poor prognosis because patients generally present at a late stage of disease and because of the limited treatment options with disappointing response rates. Patient stratification for systemic therapy in PDAC is not only beneficial for individual patients by preventing morbidity and mortality associated with treatment, it also answers to the socioeconomic issue of increasing healthcare costs together with the rise in cancer incidence worldwide. Ineffective (chemotherapy) treatment and toxicity-related complications are an expensive pitfall in the management of PDAC and must be improved on short-term.

Published studies on circulating biomarkers that can predict chemotherapy response in PDAC patients are scarce. In addition, the previously described studies are difficult to compare because of the different biomarkers investigated and variation in cut-off values (e.g. for CA19-9), study populations (LAPC and/or metastatic patients) and chemotherapy regimens. Therefore, it is hard to accept any of the studied molecules as a predictive circulating biomarker. Moreover, most of the studies lack validation of the investigated biomarkers, and the overall patient populations are small.

None of the studied circulating biomarkers mentioned in this review met the strict criteria for predictive biomarkers. No comparisons are made between a treatment group and a control group, or two different treatment groups, to determine proportions of biomarker-positive and biomarker-negative patients. Thus, the studied biomarkers cannot officially be termed as predictive. However, we believe that these studies do provide future directions for further research.

Studies investigating biomarkers specifically for response to FOLFIRINOX or gemcitabine chemotherapy are rare, despite the fact that FOLFIRINOX is currently the best treatment option for patients with locally advanced and metastatic PDAC. Four studies investigated circulating biomarkers in patients treated with FOLFIRINOX[47, 48, 62, 63] and six in patients with fluorouracil (5-FU)[38-40, 73, 76, 82] and data for these patients are combined with data from patients treated with other chemotherapeutics, like gemcitabine or nab-paclitaxel. Since FOLFIRINOX is increasingly investigated and implemented as a neoadjuvant therapy in (borderline) resectable patients, the number of patients receiving this therapy will increase, while the short-term response rate will most probably remain similar. Therefore, given the rise in application of FOLFIRINOX, stratifying patients who will benefit from this toxic treatment will be even more important in the future.

An increasing number of ongoing clinical trials are investigating potential predictive biomarkers. For example, in the ongoing Dutch iKnowIT study, blood samples are collected before and during FOLFIRINOX treatment aiming to investigate the predictive value of several biomarkers (i.e. ctDNA and miRNA) to guide FOLFIRINOX therapy. In another randomized controlled clinical trial in the Netherlands (PREOPANC-2) that compares the benefit of neoadjuvant FOLFIRINOX treatment to that of neoadjuvant gemcitabine-based chemoradiation in resectable PDAC patients, blood samples are collected at multiple time points: at baseline, during treatment and follow-up, to investigate the predictive value of circulating biomarkers. Consulting www.clinicaltrials.gov confirmed that there are several other clinical trials that include liquid biopsies in their protocol. The registered trials including the investigated biomarkers are summarized in Table 4. This is not a complete overview, since many trials are not registered and study protocols are often only known after publication.

Although this review focuses on circulating biomarkers, tumor biopsies continue to be investigated as a potential source of predictive biomarkers. Histology of tumor biopsies remains pivotal in diagnosis and tumor-specific treatment decision making. In the ongoing HALO-trial for metastasized PDAC patients, the level of hyaluronic acid (HA) in tumor samples is evaluated. Patients with high-HA tumors are randomized to the combination of gemcitabine and nab-paclitaxel only or to gemcitabine and nab-paclitaxel with an addition of PEGPH20, an enzyme that breaks down the hyaluron.[88] In the PRIMUS-001 trial, part of the Precision Panc study, genetic changes in tumor biopsies measured with Next Generation Sequencing are used to predict the efficacy of either FOLFIRINOX combined with nab-paclitaxel or gemcitabine with nab-paclitaxel.[89] Other examples are the two trials conducted by the Seoul National University Hospital and AHS Cancer Control Alberta at which the expression of *hENT1* is determined in the resected tumor material in order to decide on adjuvant gemcitabine or 5-FU (Table 4). These concepts, using

specific (genetic) changes in pancreatic tumors for tumor-specific treatment decision making, should be encouraged in other clinical trials.

The majority of the studies focused on CA19-9 as a prognostic and predictive biomarker.[36-42, 44-48, 90] The studies showed that lower pre- and posttreatment levels and decreasing levels of CA19-9 over time are associated with chemotherapy response. However, these studies presented various cut-off values and various time points of measurement. Additionally, the patient groups and the chemotherapy regimens vary among the studies, making it difficult to accept CA19-9 as a precise biomarker at this moment. Therefore, we suggest performing a patient level meta-analysis on CA19-9 to identify the best cut-off value that will facilitate using CA19-9 as a treatment predictive biomarker. This requires a large collaborative effort but may be justified because CA19-9 is currently the most commonly used biomarker in clinical practice. In addition we think that there are other cost effective markers that are easy to obtain and standardize, such as CEA, NLR, PLR, and SII that could be used to predict treatment response. Currently these markers are suggested to be used to monitor the PDAC patients in some medical centers. The rapidly evolving –omics techniques, e.g. proteomics, transcriptomics and genomics, empower discovering precise circulating biomarkers. For that reason, we think all ongoing and future clinical trials will benefit from collection of not only representative tumor biopsies, but also liquid biopsies at multiple time points before, during, and after treatment, in addition to the clinical data. However, it is improbable that a single marker will be sensitive and specific enough to be used as a solitary biomarker. A combination of various molecules and indicators is more likely to predict patient outcome and treatment effect.

CONCLUSION

Patient stratification is essential for improving the quality of life for patients and for decreasing unnecessary healthcare costs. Currently, predictive biomarkers for chemotherapy response in PDAC patients are not available. However, various potential predictive biomarkers have been investigated, and some results are promising. Ongoing and future clinical trials will benefit from structured tumor biopsies and blood sampling to investigate potential predictive biomarkers. An increasing number of clinical trials already include liquid biopsies in their study protocol in addition to tumor biopsies. Combining available clinical data to perform a meta-analysis is required for proper evaluation of existing data on promising biomarkers. It is plausible that a combination of various circulating markers is required to predict treatment response. Identification of the right circulating biomarkers in the near future is crucial for patient therapy improvement.

Table 4. Overview of PDAC clinical trials and the biomarkers to be investigated, registered at (www.clinicaltrials.gov).

Sponsor	ClinicalTrials.gov Identifier	Number of patients	Stage of disease	Treatment	Biomarker
Circulating biomarkers					
Asan Medical Center	NCT02749136	44	Borderline resectable, neoadjuvant treatment	FOLFIRINOX	Not mentioned
Massachusetts General Hospital	NCT01591733	48	Borderline resectable + resectable, neoadjuvant treatment	FOLFIRINOX + radiation	SNPs
UNC Lineberger Comprehensive Cancer Center	NCT01688336	45	Borderline unresectable + LAPC	FOLFIRINOX	CA19-9, CEA
Pancreatic Cancer Research Team	NCT01488552	60	Metastatic	Gemcitabine + nab-paclitaxel, followed by FOLFIRINOX	Miscellaneous, including CA19-9
Centre Georges Francois Leclerc	NCT03599154	100	Metastatic	Gemcitabine (+ nab-paclitaxel) FOLFIRINOX	RNA gene expression (Gemcitest)
Helse Stavanger HF	NCT02707159	70	Metastatic	Gemcitabine + nab-paclitaxel	Circulating tumor cells
National Cancer Center, Korea	NCT01333124	64	Resectable, neo-adjuvant treatment	Gemcitabine + radiation	Not mentioned
Tissue biomarkers					
Seoul National University Hospital	NCT02486497	40	Resectable, adjuvant treatment	Gemcitabine 5-FU	hENT1 protein expression
AHS Cancer Control Alberta	NCT01411072	20	Resectable, adjuvant treatment	Gemcitabine 5-FU	hENT1 protein expression
Grupo Hospital de Madrid	NCT01394120	60	Metastatic	FOLFIRINOX FOLFOX FOLFIRI Gemcitabine + capecitabine Gemcitabine Gemcitabine + erlotinib	Thymidilate Synthase, Thimidine Phosphorylase, *ERCC1* and Topoisomerase I expression
Emory University	NCT01188109	25	Resectable, adjuvant treatment	Gemcitabine + cisplatin	ERCC1 gene expression

SNP = single nucleotide polymorphism, LAPC = locally advanced pancreatic cancer, CA19-9 = carbohydrate antigen 19-9, CEA = carcinoembryonic antigen, 5-FU = fluorouracil, hENT1 = human equilibrative nucleoside transporter 1, *ERCC1* = excision repair 1, endonuclease non-catalytic subunit.

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Chapter 5

Circulating *TP53* mutations are associated with early tumor progression and poor survival in pancreatic cancer patients treated with FOLFIRINOX

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ABSTRACT

Background Biomarkers predicting treatment response may be used to stratify pancreatic ductal adenocarcinoma (PDAC) patients for therapy. The aim of this study was to identify circulating tumor DNA (ctDNA) mutations that associate with tumor progression during FOLFIRINOX chemotherapy, and overall survival (OS).

Methods Circulating cell-free DNA was analyzed with a 57 gene next-generation sequencing panel using plasma samples of 48 PDAC patients of all disease stages. Patients received FOLFIRINOX as initial treatment. Chemotherapy response was determined on CT scans as disease control (*n*=30) or progressive disease (*n*=18) within eight cycles of FOLFIRINOX, based on RECIST 1.1 criteria.

Results Detection of a *TP53* ctDNA mutation before start of FOLFIRINOX (OR 10.51, 95% CI 1.40-79.14) and the presence of a homozygous *TP53* Pro72Arg germline variant (OR 6.98, 95% CI 1.31-37.30) were predictors of early tumor progression during FOLFIRINOX in multivariable analysis. Five patients presented with the combination of a *TP53* ctDNA mutation before start of FOLFIRINOX and the homozygous Pro72Arg variant. All five patients showed progression during FOLFIRINOX. The combination of the *TP53* mutation and *TP53* germline variant was associated with shorter survival (median OS 4.4 months, 95% CI 2.6-6.2 months) compared to patients without any *TP53* alterations (median OS 13.0 months, 95% CI 8.6-17.4 months).

Conclusions The combination of a *TP53* ctDNA mutation before start of FOLFIRINOX and a homozygous *TP53* Pro72Arg variant is a promising biomarker, associated with early tumor progression during FOLFIRINOX and poor OS. The results of this exploratory study need to be validated in an independent cohort.

INTRODUCTION

Although some advances have been made in the treatment of pancreatic ductal adenocarcinoma (PDAC), the prognosis of patients remains very poor.[1, 2] The standard first-line treatment for locally advanced pancreatic cancer (LAPC) and metastatic PDAC is FOLFIRINOX chemotherapy, a combination of fluorouracil, leucovorin, irinotecan and oxaliplatin. With this treatment regimen, improved overall survival (OS) was observed in both LAPC (24.2 months vs 6-13 months)[3] and metastatic PDAC (11.1 months vs 6.8 months)[4] compared to gemcitabine chemotherapy. FOLFIRINOX is also effective in PDAC patients with stage I-II resectable or borderline resectable disease in the adjuvant setting,[5] and several clinical trials are investigating the benefit of neoadjuvant FOLFIRINOX followed by surgical resection.[6]

Despite increased survival in patient groups treated with FOLFIRINOX, only a minority of patients will show complete or partial response of the tumor,[7, 8] while approximately 20-30% already develop progressive disease during FOLFIRINOX.[4, 9] Unfortunately, 60-70% of patients experiences severe, grade ≥4 toxicity from FOLFIRINOX.[3, 4, 8] Biomarkers could stratify patients for available therapies. Especially biomarkers that can be easily measured in the circulation, as opposed to tumor tissue, would be ideal. Such a predictive biomarker could prevent non-responding patients from FOLFIRINOX-induced toxicity and select these patients for other treatment.

Circulating cell-free DNA (ccfDNA), including circulating tumor DNA (ctDNA), are short fragments of DNA released into the bloodstream after apoptosis and necrosis of (tumor) cells. ctDNA can be detected in blood serum or plasma and the presence of tumor mutations in ctDNA is a poor prognostic factor in PDAC patients.[10-12] Moreover, increasing ctDNA levels over time and the detection of new mutations during chemotherapy are associated with progression of disease.[13-15] However, most studies focus on *KRAS* mutations only,[13, 15] while several other known cancer-associated gene mutations may indicate PDAC progression and treatment response as well.

The aim of this pilot study was to investigate the value of ctDNA mutations in PDAC patients, detected before the start of treatment or after only one cycle of chemotherapy, to predict early tumor progression during FOLFIRINOX and their association with OS.

MATERIALS AND METHODS

This article was written according to the Reporting recommendations for tumor marker prognostic studies (REMARK) guidelines.[16]

Patient selection

All patients were selected from two multicenter, prospective trials in the Netherlands. Patients with resectable or borderline resectable PDAC participated in the randomized clinical trial PREOPANC-2 (Dutch trial register NL7094) comparing neoadjuvant FOLFIRINOX to neoadjuvant gemcitabine-based chemoradiotherapy, followed by surgical resection of the primary tumor if applicable. Patients with locally advanced or metastatic PDAC were selected from the prospective cohort study iKnowIT (Dutch trial register NL7522) focusing on the predictive value of circulating biomarkers. Both trials were approved by the ethics committees of all participating hospitals with patients included in this article: Erasmus Medical Center Rotterdam (MEC-2018-087 and MEC-2018-004), Amsterdam UMC, location Academic Medical Center (2018_196 and 2018_138), Leiden University Medical Center (L18.070 and L18.053), Isala hospital Zwolle (180606), and Medisch Spectrum Twente Enschede (H18-081). All patients provided written informed consent and both studies were conducted in accordance with the declaration of Helsinki.

Due to the explorative character of this study, no formal sample size calculation was performed. The authors estimated a sample size of 48 to be achievable and sufficient to draw conclusions from this pilot study. Patient samples were not selected consecutively, but based on the availability of plasma samples and treatment response outcome, in order to have a sufficient number of patients in both investigational groups.

After histological confirmation of the primary tumor and/or metastases, patients from all PDAC disease stages received initial treatment with FOLFIRINOX between February 2018 and September 2019. Patients with resectable, borderline resectable, or locally advanced disease received a maximum of eight cycles of FOLFIRINOX, whereas patients with metastatic disease received a maximum of 12 cycles. Exclusion criteria for patient selection were: age under 18 years, co-treatment with other chemotherapeutics, and previous treatment with FOLFIRINOX. After each fourth cycle of chemotherapy, a CT scan was performed to evaluate response to treatment according to the Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria,[17] as part of standard clinical practice. In case of progressive disease, FOLFIRINOX was discontinued. Disease control was defined as stable disease, partial or complete response, and these patients would continue FOLFIRINOX as planned. Patient characteristics, such as age, sex, stage of disease, laboratory

results, CT scan evaluations, and follow-up data were retrieved from medical records by a medical doctor. Follow-up ended upon the death of the patient.

Sample collection

Peripheral venous blood samples were collected before the start of FOLFIRINOX and two weeks after the first cycle of FOLFIRINOX. Blood was collected in 10 mL EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) in the Erasmus Medical Center, or 10 mL CellSave tubes (Menarini Silicon Biosystems, Castel Maggiore, Italy) in other centers. CellSave tubes preserve circulating tumor cells and ctDNA up to 96 hours at room temperature. CellSave tubes were transferred to the Erasmus Medical Center for processing.

DNA isolation

Plasma was isolated within 4 hours of collection for EDTA tubes or within 72 hours for CellSave tubes. For plasma separation, tubes were centrifuged at 1000g for 10 minutes and again at 1700g for 10 minutes after transfer into new tubes. Plasma was stored at -80 °C until further use. ccfDNA was isolated from 3 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was eluted in 30 µL buffer AVE (RNase-free water with 0.04% sodium azide) and the eluate was re-applied twice to optimize the DNA yield.

Next-generation sequencing

ccfDNA concentrations were measured with RT-qPCR quantification using Alu115 primer pairs (Swift Biosciences, Ann Arbor, MI, USA).[18] The ccfDNA concentrations were derived from the Alu115 RT-qPCR results, representing the total quantity of usable ccfDNA, but excluding short fragments as a result of DNA degradation.

ccfDNA was sequenced using the Accel-Amplicon 57G Plus Pan-Cancer Profiling Panel (Swift Biosciences, Ann Arbor, MI, USA) which covers 286 amplicons of 57 genes. A full gene list is provided in Supplementary Table 1. DNA libraries were prepared using 3-10 ng DNA input, depending on the maximum ccfDNA concentration available. DNA libraries were prepared by multiplex PCR, amplified for 25 cycles in total, followed by the ligation of Illumina adaptors with sample-specific indices. These libraries were pooled and sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA), paired-end, with reads of 150 base pairs in length. Fastq files were uploaded in the online Genialis software platform (Genialis, Houston, TX, USA) to trim adaptors of the read and to align the reads to the Human Genome hg19 and to perform LoFreq variant-calling. As a control, four DNA samples were included to assess the consistency of mutation detection: Quantitative Multiplex DNA Reference Standard HD701 (Horizon Discovery, Waterbeach, UK), ccfDNA isolated from ctDNA Ref v2 AF2% plasma (Seraseq Gaithersburg, MD, USA),

ccfDNA isolated from ctDNA Ref v2 WT plasma (Seraseq, Gaithersburg, MD, USA), and ccfDNA isolated from plasma of a patient diagnosed with lung carcinoma and previously sequenced with IonTorrent sequencing method (ThermoFisher Scientific, Waltham, MA, USA). For mutation calling, several criteria were used: only non-synonymous mutations with variant allele frequency >1%, quality score >200, ≥10 reads in total and ≥5 reads per strand, and Fisher strand bias <100 were called mutations. Mutations with allele frequencies ~50% (heterozygous) and ~100% (homozygous) were considered germline mutations, not ctDNA mutations. All base changes and accompanying amino acid changes were annotated on the forward strand.

Statistical analyses

Continuous data with a non-normal distribution were compared with either Mann-Whitney U tests, or with Wilcoxon Signed Rank tests for paired data. Categorical data, such as detection rates of mutations, were compared using Fisher's exact or Pearson's Chi-squared tests where appropriate.

Univariable and multivariable binary logistic regression was performed to analyze the predictive value of ccfDNA concentrations and ctDNA mutations for tumor progression during FOLFIRINOX chemotherapy, adjusted for known predictive patient characteristics: stage of disease, and CA19-9 levels. Multicollinearity between variables was checked using the variance inflation factor (VIF). Factors with $P<0.10$ and VIF<3 were selected for multivariable analysis.

OS was calculated as the time between the start of FOLFIRINOX and death. All patients included in this cohort died of cancer progression. Differences in median OS were derived from Kaplan-Meier curves whereby groups were compared using log-rank tests. The prognostic value of ctDNA mutations was also tested with univariable and multivariable Cox regression analyses, including known prognostic factors: age, stage of disease, and CA19-9 levels. Multicollinearity between variables was checked using the variance inflation factor (VIF). Factors with $P<0.10$ and VIF<3 were selected for multivariable analysis.

Only two-sided tests were used and *P*-values <0.05 were considered statistically significant. Data were analyzed using SPSS Statistics for Windows (version 25.0; IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

In total, ccfDNA isolated from plasma from 48 patients was sequenced both before and after the first cycle of FOLFIRINOX, resulting in 96 samples. Eighteen patients had resectable or borderline resectable disease, 16 LAPC, and 14 metastatic disease. Of these patients, 18 (37.5%) showed progressive disease during FOLFIRINOX, as presented in Table 1. The progressive disease patient group consisted of 6 patients with resectable disease, 6 patients with LAPC and 6 patients with metastatic disease. The majority of patients had no detectable ctDNA mutations before (31/48 = 64.6%) or after one cycle of FOLFIRINOX (38/48 = 79.2%).

ctDNA detection

In disease control patients ccfDNA concentrations increased from 6.32 ng/mL plasma (IQR 4.15-14.73 ng/mL plasma) before start of FOLFIRINOX to 14.25 ng/mL plasma (IQR 8.57-21.58 ng/mL plasma, *P*=0.028) after one cycle of FOLFIRINOX. In progressive disease patients concentrations increased from 5.54 ng/mL plasma (IQR 1.95-8.96 ng/mL plasma) before start of FOLFIRINOX to 7.52 ng/mL plasma (IQR 3.13-16.72 ng/mL plasma, *P*=0.007) after one cycle of FOLFIRINOX, as presented in Supplementary Figure 1. There was no statistically significant difference in ccfDNA concentration before chemotherapy between disease control and progressive disease patients (*P*=0.074). After one cycle of chemotherapy, the median ccfDNA concentration was significantly lower in patients with progressive disease (*P*=0.018) (Supplementary Figure 1).

In 27 out of 96 (28.1%) of the sequenced plasma samples, at least one ctDNA mutation was detected, corresponding to samples from 21 out of 48 patients. Supplementary Figure 2 shows an overview of each ctDNA mutation detected per plasma sample in 21 out of 48 patients, including their variant allele frequency (VAF).

ctDNA mutation detection rates did not differ between samples collected before start of FOLFIRINOX (35.4%) and samples collected after one cycle of FOLFIRINOX (20.8%, *P*=0.112). There were no differences in ctDNA mutation detection rates before start of FOLFIRINOX between patients with disease control (30.0%) and patients with progressive disease (44.4%, *P*=0.361), or after 1 cycle of chemotherapy between patients with disease control (16.7%) and patients with progressive disease (27.8%, *P*=0.468).

The most frequently detected ctDNA mutations were *KRAS* (17/96 samples in total, 12/48 before chemotherapy, 5/48 after 1 cycle of chemotherapy), *TP53* (12/96 samples in total, 8/48 before chemotherapy, 4/48 after 1 cycle of chemotherapy), and *PIK3CA* (4/96

Table 1. Patient characteristics.

	All patients, *n*=48 (%)
Age (years), mean (range)	64 (41-78)
Sex	
Male	28 (58.3)
Female	20 (41.7)
Stage of disease	
Resectable	18 (37.5)
Locally advanced	16 (33.3)
Metastatic	14 (29.2)
Response* to FOLFIRINOX	
Stable disease	24 (50.0)
Partial response	6 (12.5)
Complete response	0 (0)
Progressive disease	18 (37.5)
Response* to FOLFIRINOX, dichotomized	
Disease control	30 (62.5)
Progressive disease	18 (37.5)
Time point of CT evaluation progressive disease* (*n*=18)	
After cycle 1	1 (5.6)
After cycle 2	1 (5.6)
After cycle 3	2 (11.1)
After cycle 4	10 (55.6)
After cycle 8	4 (22.2)
Number of cycles of FOLFIRINOX received, mean (range)	7 (1-12)
Baseline CA19-9 (kU/L), median (IQR)	369 (66-2015)
DNA concentration before the start of FOLFIRINOX (ng/mL plasma), median (IQR)	5.98 (3.59-13.67)
DNA concentration after one cycle of FOLFIRINOX (ng/mL plasma), median (IQR)	11.52 (6.42-18.31)
Number of tumor mutations detected before the start of FOLFIRINOX	
0	31 (64.6)
1	9 (18.8)
2	4 (8.3)
3	4 (8.3)
Number of tumor mutations detected after one cycle of FOLFIRINOX	
0	38 (79.2)
1	7 (14.6)
2	2 (4.2)
3	1 (2.1)

CA19-9 = carbohydrate antigen 19-9, IQR = interquartile range. *According to the RECIST 1.1 criteria.

samples in total, 2/48 before chemotherapy, 2/48 after 1 cycle of chemotherapy) mutations. Differences in detection rates between patients with disease control and patients with progressive disease are presented in Table 2. Before start of FOLFIRINOX *TP53* ctDNA mutations were significantly more often detected in patients with progressive disease (33.3%) compared to patients with disease control (6.7%, *P*=0.040). After chemotherapy no statistically significant differences between disease control and progressive patients were found in detection rates of any of the major tumor mutations. For this reason, only results retrieved from samples collected before FOLFIRINOX will be further discussed. There were no differences in detection rates of ctDNA mutations between the different stages of disease (Supplementary Table 2).

Table 2. Differences in circulating tumor DNA (ctDNA) mutation detection rates between patients with disease control and patients with progressive disease during FOLFIRINOX. *P*-values were calculated with Fisher's exact tests.

	Disease control patients, *n*=30 (%)	**Progressive disease patients, *n*=18 (%)**	***P***	**All patients, *n*=48 (%)**
ctDNA mutations detected before the start of FOLFIRINOX				
Any ctDNA mutation	9 (30.0)	8 (44.4)	0.361	17 (35.4)
KRAS	6 (20.0)	6 (33.3)	0.325	12 (25.0)
TP53	2 (6.7)	6 (33.3)	0.040	8 (16.7)
PIK3CA	1 (3.3)	1 (5.6)	1.000	2 (4.2)
ctDNA mutations detected after one cycle of FOLFIRINOX				
Any ctDNA mutation	5 (16.7)	5 (27.8)	0.468	10 (20.8)
KRAS	2 (6.7)	3 (16.7)	0.349	5 (10.4)
TP53	1 (3.3)	3 (16.7)	0.142	4 (8.3)
PIK3CA	0 (0)	2 (11.1)	0.136	2 (4.2)

Germline variant detection

Five germline variants were found in multiple patients: *TP53* p.Pro72Arg, *KDR* p.Gln472His, *KIT* p.Met541Leu, *ERBB2* p.Ile625Val, and *PIK3CA* p.Ile391Met. All these germline variants are known single nucleotide polymorphisms (SNPs). In Table 3, the frequencies of all genotypes are presented per response group: disease control or progressive disease. There was no difference in the distribution of the different genotypes (homozygous reference allele, heterozygous, or homozygous mutant allele) between patients with disease control and patients with progressive disease for the germline mutations in *KDR* (*P*=0.955), *KIT* (*P*=0.932), *ERRBB2* (*P*=0.521) and *PIK3CA* (*P*=0.624). The homozygous *TP53* Pro72Arg variant was more often detected in patients with progressive disease (83.3%) compared to disease control patients (50.0%, *P*=0.031). There were no differences in the distribution of germline variant genotypes between the different stages of disease (Supplementary Table 2).

Table 3. Differences in genotype frequencies of single nucleotide polymorphisms (SNPs) in patients with disease control and patients with progressive disease during FOLFIRINOX. *P*-values were calculated with Pearson's Chi-squared tests.

	Disease control patients, *n*=30 (%)	**Progressive disease patients, *n*=18 (%)**	***P***	**All patients, *n*=48 (%)**
TP53 Pro72Arg				
Pro/Pro	2 (6.7)	1 (5.6)	0.056	3 (6.3)
Pro/Arg	13 (43.3)	2 (11.1)		15 (31.3)
Arg/Arg	15 (50.0)	15 (83.3)		30 (62.5)
Pro/Pro + Pro/Arg	15 (50.0)	3 (16.7)	0.031	18 (37.5)
Arg/Arg	15 (50.0)	15 (83.3)		30 (62.5)
KDR Gln472His				
Gln/Gln	18 (60.0)	10 (55.6)	0.955	28 (58.3)
Gln/His	9 (30.0)	6 (33.3)		15 (31.3)
His/His	3 (10.0)	2 (11.1)		5 (10.4)
KIT Met541Leu				
Met/Met	24 (80.0)	14 (77.8)	0.932	38 (79.2)
Met/Leu	5 (16.7)	3 (16.7)		8 (16.7)
Leu/Leu	1 (3.3)	1 (5.6)		2 (4.2)
ERBB2 Ile625Val				
Ile/Ile	18 (60.0)	9 (50.0)	0.521	27 (56.3)
Ile/Val	11 (36.7)	7 (38.9)		18 (37.5)
Val/Val	1 (3.3)	2 (11.1)		3 (6.3)
PIK3CA Ile391Met				
Ile/Ile	26 (86.7)	17 (94.4)	0.624	43 (89.6)
Ile/Met	3 (10.0)	1 (5.6)		4 (8.3)
Met/Met	1 (3.3)	0 (0)		1 (2.1)

Arg = arginine, Gln = glutamine, His = histidine, Ile = isoleucine, Leu = leucine, Met = methionine, Pro = proline, Val = valine.

Predictive value of circulating mutations

Detection of *TP53* ctDNA mutations before start of chemotherapy (odds ratio (OR) 7.00, 95% CI 1.23-39.78, *P*=0.028) and the presence of a homozygous *TP53* Pro72Arg germline SNP (OR 5.00, 95% CI 1.20-20.92, *P*=0.028) were predictive factors of progression during FOLFIRINOX (Table 4). There was no collinearity between these factors (variance inflation factor 1.0). *TP53* mutations remained significant predictors of tumor progression during FOLFIRINOX after adjusting for stage of disease and baseline CA 19-9 level: OR 10.51 (95% CI 1.40-79.14, *P*=0.022) for detection of *TP53* ctDNA mutations before start chemotherapy and OR 6.98 (95% CI 1.31-37.30, *P*=0.023) for a homozygous *TP53* Pro72Arg variant presence.

Table 4. Univariable and multivariable binary logistic regression model for the prediction of early tumor progression during FOLFIRINOX.

	Univariable		Multivariable	
Variable	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Stage of disease				
Resectable	Ref			
LAPC	1.20 (0.29-4.91)	0.800		
Metastatic	1.50 (0.36-6.35)	0.582		
CA19-9 at baseline (per 100 kU/L)	1.01 (0.99-1.02)	0.280		
ctDNA mutation detected before start FOLFIRINOX				
No	Ref			
Yes	1.87 (0.55-6.29)	0.314		
KRAS ctDNA mutation detected before start FOLFIRINOX				
No	Ref			
Yes	2.00 (0.53-7.54)	0.306		
TP53 ctDNA mutation detected before start FOLFIRINOX				
No	Ref		Ref	
Yes	7.00 (1.23-39.78)	0.028	10.51 (1.40-79.14)	0.022
TP53 Pro72Arg germline variant				
Not homozygous (Pro/Pro or Pro/Arg)	Ref		Ref	
Homozygous (Arg/Arg)	5.00 (1.20-20.92)	0.028	6.98 (1.31-37.30)	0.023
KDR Gln472His germline variant				
No (Gln/Gln)	Ref			
Yes (Gln/His or His/His)	1.20 (0.37-3.91)	0.762		
KIT Met541Leu germline variant				
No (Met/Met)	Ref			
Yes (Met/Leu or Leu/Leu)	1.14 (0.27-4.76)	0.854		
ERBB2 Ile625Val germline variant				
No (Ile/Ile)	Ref			
Yes (Ile/Val or Val/Val)	1.50 (0.46-4.87)	0.500		
PIK3CA Ile391Met germline variant				
No (Ile/Ile)	Ref			
Yes (Ile/Met or Met/Met)	0.38 (0.04-3.72)	0.408		

Arg = arginine, CA19-9 = carbohydrate antigen 19-9, CI = confidence interval, Gln = glutamine, His = histidine, Ile = isoleucine, LAPC = locally advanced pancreatic cancer, Leu = leucine, Met = methionine, OR = odds ratio, Pro = proline, Ref = reference value, Val = valine.

Five patients (out of the total cohort of *n*=48, 10.4%) had both the *TP53* ctDNA mutation before the start of FOLFIRINOX as well as the homozygous Pro72Arg variant present. All five of these patients showed progression during FOLFIRINOX. The combination of both

TP53 mutations detected before the start of FOLFIRINOX showed a sensitivity of 27.8% and specificity of 100% to predict tumor progression during FOLFIRINOX in this cohort. The positive predictive value was 100% and the negative predictive value 69.8%.

Prognostic value of circulating mutations

Out of 48 patients, 33 (69%) died during follow-up. The median follow-up of patients alive at last follow-up was 16.8 months.

Kaplan-Meier curves are shown in Figure 1. Patients with *TP53* ctDNA mutations detected before the start of FOLFIRINOX showed a median OS of 5.6 months (95% CI 3.9-7.2 months). Patients without *TP53* ctDNA mutations had a median OS of 14.5 months (95% CI 11.6-17.3 months, $P<0.001$), as presented in Figure 1A. Patients with a homozygous *TP53* Pro72Arg variant did not show significantly worse OS (median OS 10.7 months, 95% CI 8.3-13.1 months) compared to patients without this homozygous SNP (median OS 13.0 months, 95% CI 3.6-22.4 months, $P=0.285$), shown in Figure 1B.

The combination of the presence of a circulating *TP53* ctDNA mutation before the start of FOLFIRINOX with a homozygous *TP53* Pro72Arg germline variant was associated with shorter OS (median OS 4.4 months, 95% CI 2.6-6.2 months) compared to patients without this combination (median OS 13.0 months, 95% CI 8.6-17.4 months, $P<0.001$) (Figure 1C). Patients with both a *TP53* ctDNA mutation and a homozygous *TP53* Pro72Arg germline variant detected before the start of FOLFIRINOX showed similar OS (median OS 4.4 months; 95% CI 2.6-6.2 months) compared to patients with *TP53* ctDNA mutations alone (median OS 5.9 months; 95% CI 5.4-6.4 months, $P=0.237$) (Figure 1D). Patients with any ctDNA mutation before the start of FOLFIRINOX, including *TP53* and *KRAS* mutations, did as well show shorter OS (median OS 6.6 months, 95% CI 5.2-8.1 months) compared to patients without any detectable ctDNA mutation (median OS 15.7 months, 95% CI 13.0-18.3 months, $P<0.001$) (Figure 1E). Patients with a *KRAS* ctDNA mutation detected before the start of FOLFIRINOX showed worse OS (median OS 5.9 months, 95% CI 4.2-7.6 months) than patients without a *KRAS* ctDNA mutation (median OS 14.5 months, 95% CI 12.4-16.5 months, $P=0.002$) (Figure 1F).

A univariable and multivariable model for OS is presented in Table 5. A *TP53* ctDNA mutation detected before start of FOLFIRINOX was a significant predictor for OS in univariable analysis (HR 4.39. 95% CI 1.90-10.13, $P<0.001$), but not in multivariable analysis.

The presence of any detectable ctDNA mutations before start of FOLFIRINOX remained a significant prognostic factor for OS after adjusting for age, stage of disease, and baseline CA 19-9 level with HR 4.29 (95% CI 1.40-13.12, *P*=0.011). For the presence of the *TP53* Pro72Arg germline variant, prognostic value could not be demonstrated.

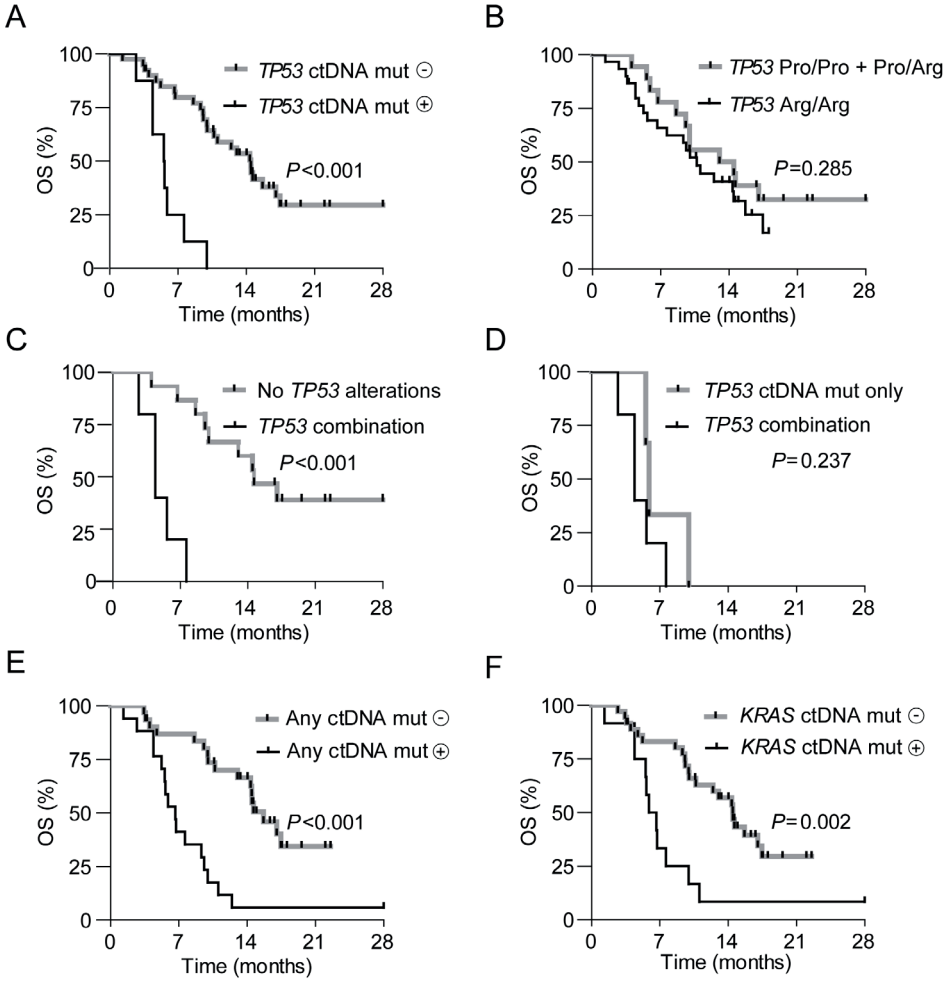

Figure 1. Kaplan-Meier curves for overall survival (OS) for patients with and without circulating mutations detected before the start of FOLFIRINOX.

(A) Patients with (*n*=8) or without (*n*=40) a *TP53* ctDNA mutation. (B) Patients with (*n*=30) and without (*n*=18) a homozygous germline *TP53* Pro72Arg variant. (C) Patients with the combination of a *TP53* ctDNA mutation and a homozygous germline *TP53* Pro72Arg variant (*n*=5) and patients without a *TP53* ctDNA mutation and without a homozygous *TP53* Pro72Arg variant (*n*=15). (D) Patients with the combination of *TP53* mutations (*n*=5) compared to patients with a *TP53* ctDNA mutation alone (*n*=3). (E) Patients with (*n*=17) and without (*n*=31) any ctDNA mutation. (F) Patients with (*n*=12) and without (*n*=32) a *KRAS* ctDNA mutation. *P*-values were calculated with log-rank tests. Mut = mutation.

Table 5. Univariable and multivariable Cox proportional hazards model for overall survival (OS) after FOLFIRINOX chemotherapy.

	Univariable		Multivariable	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age (per year)	1.00 (0.96-1.05)	0.932		
Stage of disease				
Resectable	Ref		Ref	
LAPC	1.22 (0.51-2.94)	0.659	1.06 (0.42-2.69)	0.905
Metastatic	2.77 (1.20-6.39)	0.017	1.61 (0.65-4.02)	0.308
CA19-9 at baseline (per 100 kU/L)	1.00 (1.00-1.01)	0.041	1.00 (1.00-1.00)	0.544
ctDNA mutation detected before start chemotherapy				
No	Ref		Ref	
Yes	4.22 (2.04-8.75)	<0.001	4.29 (1.40-13.12)	0.011
KRAS ctDNA mutation detected before start chemotherapy				
No	Ref		Ref	
Yes	3.16 (1.48-6.71)	0.003	0.48 (0.11-1.99)	0.308
TP53 ctDNA mutation detected before start chemotherapy				
No	Ref		Ref	
Yes	6.26 (2.47-15.87)	<0.001	3.30 (0.78-13.92)	0.104
TP53 Pro72Arg germline variant				
Not homozygous (Pro/Pro or Pro/Arg)	Ref			
Homozygous (Arg/Arg)	1.47 (0.72-3.02)	0.289		
KDR Gln472His germline variant				
No (Gln/Gln)	Ref			
Yes (Gln/His or His/His)	0.73 (3.59-1.50)	0.395		
KIT Met541Leu germline variant				
No (Met/Met)	Ref			
Yes (Met/Leu or Leu/Leu)	1.81 (0.83-3.92)	0.134		
ERBB2 Ile625Val germline variant				
No (Ile/Ile)	Ref			
Yes (Ile/Val or Val/Val)	0.96 (0.48-1.91)	0.900		
PIK3CA Ile391Met germline variant				
No (Ile/Ile)	Ref			
Yes (Ile/Met or Met/Met)	1.07 (0.38-3.06)	0.893		

Arg = arginine, CA19-9 = carbohydrate antigen 19-9, CI = confidence interval, ctDNA = circulating tumor DNA, Gln = glutamine, HR = hazard ratio, His = histidine, Ile = isoleucine, LAPC = locally advanced pancreatic cancer, Leu = leucine, Met = methionine, OS = overall survival, Pro = proline, Ref = reference value, Val = valine.

DISCUSSION

This multicenter pilot study describes the predictive and prognostic value of ctDNA mutations in PDAC patients, detected with NGS before and after one cycle of FOLFIRINOX. We found that circulating *TP53* mutations detected before the start of FOLFIRINOX predict tumor progression during FOLFIRINOX. These mutations include both *TP53* ctDNA mutations and a homozygous *TP53* Pro72Arg germline variant. Furthermore, circulating *TP53* mutations were found to be a poor prognostic factor for OS in PDAC patients treated with FOLFIRINOX. The results of our study suggest that PDAC patients could be spared from ineffective FOLFIRINOX and its side effects by a simple blood draw before the start of treatment.

To our knowledge, *TP53* ctDNA mutations and the common *TP53* Pro72Arg variant have not previously been described for their predictive value for FOLFIRINOX response in PDAC. However, both types of mutations have been described to play a role in cancer, including PDAC development and progression.[19-21]

It is important to distinguish the prognostic and predictive value of ctDNA mutations from tumor tissue-specific mutations. *KRAS* mutations are present in almost all PDAC tumors, and ~40% of PDAC tumors have *TP53* mutations.[22] Only a limited number of the mutant PDAC patients, however, have detectable ctDNA mutations, in our cohort: 44%. It is known that PDAC patients with mutant *KRAS* and mutant *TP53* tumors have a worse prognosis compared to patients with wild-type *KRAS* and *TP53* tumors.[23, 24] The prognosis is even worse for patients with detectable ctDNA mutations. The underlying hypothesis for this negative prognostic effect of mutant ctDNA is that aggressive tumors with extensive metastatic properties grow faster, have a high cell death rate, infiltrate (large) blood vessels and lead to a higher tumor load, thus shed more ctDNA into the circulation.[25] The prognostic value of detectable ctDNA mutations in patients with PDAC has been described in several studies. The presence of any and/or specific ctDNA mutations, such as *KRAS* and *TP53,* are associated with poor OS and progression-free survival.[26-28] Our results support this hypothesis: the detection of any ctDNA mutations before the start of FOLFIRINOX was a negative prognostic factor for OS (HR 4.29, 95% CI 1.40-13.12, *P*=0.011). Moreover, all patients with a detectable *TP53* ctDNA mutation before the start of FOLFIRINOX died from PDAC progression within 10 months. In our cohort, *KRAS* ctDNA mutations were associated with OS in univariable analyses, but not statistically significant in multivariable analyses.

The *TP53* gene is an important tumor suppressor gene. Wild-type *TP53* regulates the cell cycle, initiates apoptosis and senescence, and activates DNA repair in situations

of DNA damage and cellular stress, thus inhibiting tumorigenesis.[29] *TP53* is the second most frequently mutated gene in PDAC and is likely responsible for the susceptibility to cancer development.[22] In human cancers, missense mutations in *TP53* are the most common type, often leading to gain-of-function and promotion of tumorigenesis.[29, 30] Most somatic *TP53* mutations are located in codons 175, 245, 248, 249, 273, and 282.[30, 31] Others have shown that restoration of wild-type *TP53* in PDAC cell lines with gain-of-function *TP53* mutations enhances the sensitivity to 5-FU, irinotecan, cisplatin, and gemcitabine.[32] These data support the results of our study: patients without detectable *TP53* ctDNA mutations showed a better response to FOLFIRINOX.

The germline *TP53* Pro72Arg SNP is a well-known variant in the human population. With the replacement of a guanine base by a cytosine base, the accompanying amino acid changes from a proline (Pro) into an arginine (Arg). This amino acid change affects the structure of the protein and might thereby influence its function.[33] The *TP53* Pro72Arg variant shows varying allele frequencies in different populations, according to the 1000 genomes project.[34] Because of its high frequency in humans, the SNP *TP53* Pro72Arg has been studied for its association with cancer risk and cancer development in a multitude of studies.[19] Allele frequencies of this SNP are known to be different in European compared to Asian populations and the combination with ethnicity-specific genetic makeup could lead to different phenotypes. In our Dutch Caucasian PDAC cohort the Arg/Arg variant is most prevalent: 62.5% of patients show this homozygous variant. Since no healthy controls were included in this experiment, we were not able to assess whether this frequency is different from the healthy Dutch population, and whether the Arg variant is associated with an increased risk of PDAC. Though, we believe that for the interpretation of clinical trials it is very important to keep in mind that genotypes might influence the response to treatment. It cannot be assumed that results are directly applicable to patients from other ethnicities or elsewhere in the world since allele frequencies of SNPs might differ considerably.

The combination of the *TP53* Pro72Arg variant with a somatic *TP53* mutation in tumor tissues has been described for its poor prognostic value in other cancer types,[35] however not yet in PDAC. It would be relevant to further analyze whether in this population more poor prognostic features could be found with the use of large public databases.

The variety in ctDNA mutation detection methods between different study results makes it difficult to draw general conclusions. For example, Droplet Digital PCR has a higher sensitivity for ctDNA mutation detection than NGS, but can only be used to search for pre-determined specific mutations, such as in *KRAS* codons 12 and 13.[36] We used a broad

57 gene cancer panel, including more amplicons than only the major PDAC hotspots and covering the entire *TP53* gene.

For this exploratory study, we included patients with PDAC from all disease stages. We specifically chose this study design for a couple of reasons. First, the distinction between disease stages is often difficult and we believe that the disease stage determined with radiography is a less important factor for the choice of treatment and prognostic outcomes than the molecular biology of PDAC. Second, with the increasing number of clinical trials investigating neoadjuvant (FOLFIRINOX) chemotherapy, including resectable PDAC patients in biomarker studies is required for future personalized treatment. However, we do acknowledge that including patients from different disease stages is also a limitation of the study. Treatment schedules differ between stages of disease. Resectable patients might undergo surgical resection of the tumor, and LAPC patients sometimes receive additional stereotactic body radiation therapy. Although our data does not show differences in detection rates of ctDNA mutations or germline variants between the different disease stages, treatment schedules might have impact on survival differences between or even within disease stages.

The low amount of ccfDNA/ctDNA in PDAC patients compared to patients with other solid cancers, such as lung cancer,[37] is another important limitation when using broader sequencing techniques. Furthermore, the sensitivity to detect ctDNA mutations using NGS in this study can be improved. It is important, however, to emphasize the stringent method we used to measure ccfDNA concentrations. Instead of the more commonly used, less accurate methods, such as NanoDrop (spectrophotometric) or Qubit (fluorometric), we have used RT-qPCR with Alu115 primers to determine the true ccfDNA concentration, preventing overestimation of ccfDNA quantity.[38] Moreover, due to the study design of this pilot study, including a relative small number of patients, we did not consecutively select patients for NGS.

This study was designed as a broad, exploratory pilot study, since there is limited literature on possible predictive biomarkers for FOLFIRINOX response in patients with PDAC.[39] The next step would be to conduct a validation study including a larger patient cohort, focused on *TP53* mutations alone, comparing different treatment regimens to FOLFIRINOX and implementing a more sensitive NGS protocol. By increasing the amount of plasma for DNA isolation, the ccfDNA yield will increase. A higher DNA input amount for sequencing library preparation would increase the probability to detect rare tumor mutations. Last, a molecular barcoding technique with unique molecular identifiers (UMIs) could be used to be able to detect ctDNA mutations at lower allele frequencies without the risk of false positive mutation calling, because errors introduced

during library preparation, target enrichment, or sequencing can be easily filtered out.[40] When including more patients, subgroup analyses on the different tumor stages can be performed in order to check if the predictive value is the same in all PDAC patients receiving FOLFIRINOX. It would be important to see whether treatment response can also be predicted with circulating *TP53* mutations for other types of chemotherapy in PDAC, and if patients that are not responding to FOLFIRINOX could benefit from, for example, gemcitabine-based chemotherapy.

In summary, the combination of a *TP53* ctDNA mutation with a homozygous *TP53* Pro72Arg germline variant is a marker for early tumor progression during FOLFIRINOX and is associated with poor OS. Before translating these results to clinical practice and adjusting treatment decisions, additional cohort studies will be necessary to validate our findings.

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SUPPLEMENTARY FILES

Supplementary Table 1. Genes included in Accel-Amplicon 57G Plus Pan-Cancer Profiling Panel.

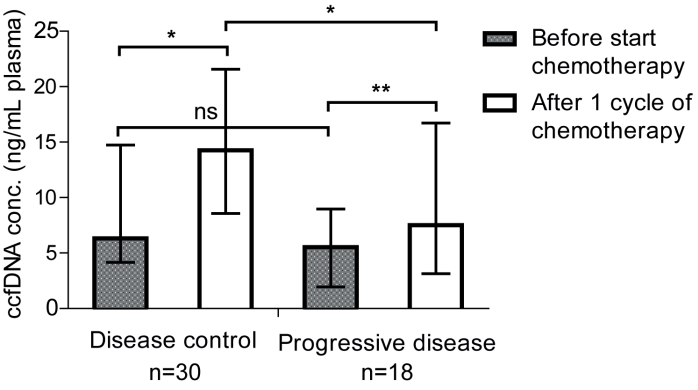
ABL1	*EZH2*	*JAK3*	*PTPN11*
AKT1	*FBXW7*	*KDR*	*RB1*
ALK	*FGFR1*	*KIT*	*RET*
APC	*FGFR2*	*KRAS*	*SMAD4*
ATM	*FGFR3*	*MAP2K1*	*SMARCB1*
BRAF	*FLT3*	*MET*	*SMO*
CDH1	*FOXL2*	*MLH1*	*SRC*
CDKN2A	*GNA11*	*MPL*	*STK11*
CSF1R	*GNAQ*	*MSH6*	*TP53**
CTNNB1	*GNAS*	*NOTCH1*	*TSC1*
DDR2	*HNF1A*	*NPM1*	*TSC2*
DNMT3A	*HRAS*	*NRAS*	*VHL*
EGFR	*IDH1*	*PDGFRA*	
ERBB2	*IDH2*	*PIK3CA*	
ERBB4	*JAK2*	*PTEN*	

*Full exon coverage

Supplementary Table 2. Differences in circulating tumor DNA (ctDNA) mutation and germline variant detection rates between patients with different disease stages. *P*-values were calculated with Pearson's Chi-squared tests.

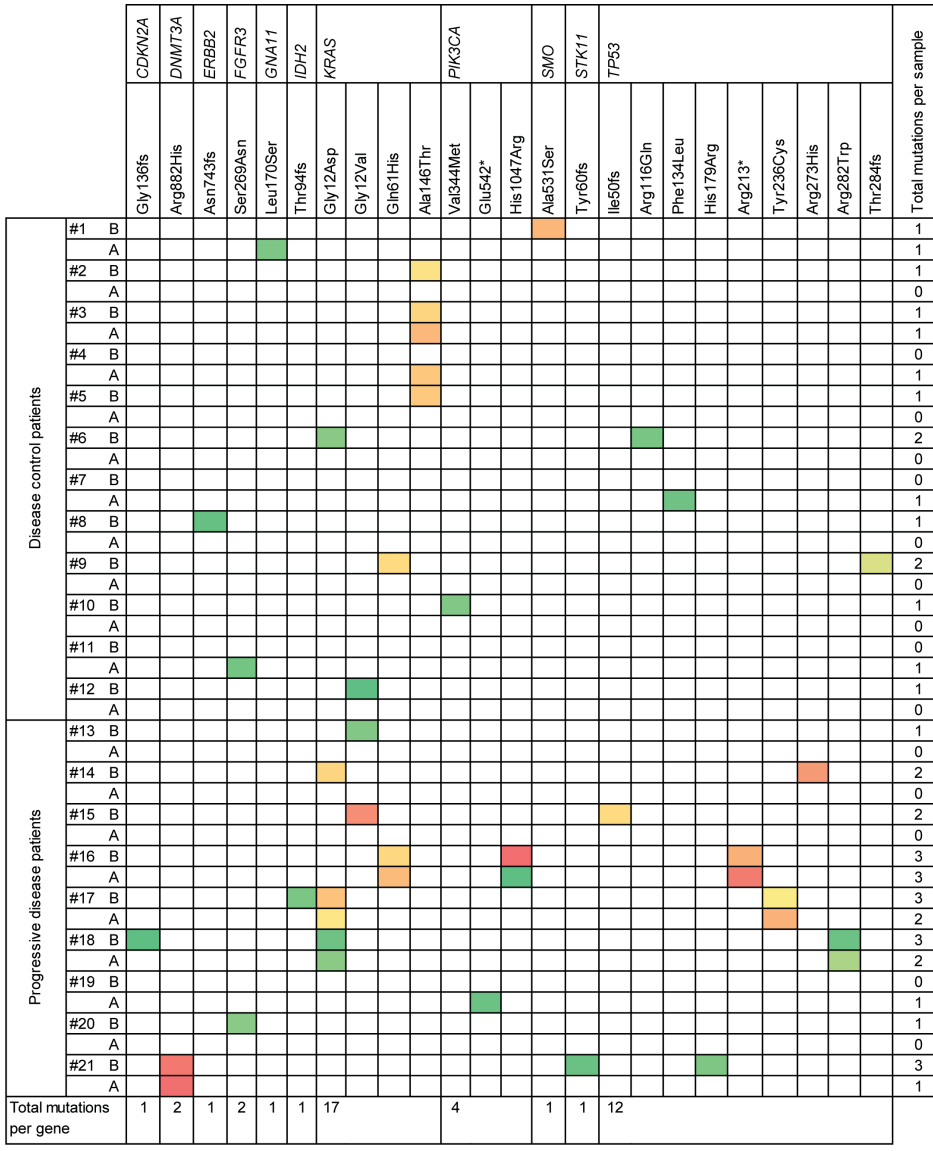
	Resectable disease patients *n*=18 (%)	**Locally advanced patients *n*=16 (%)**	**Metastatic disease patients *n*=14 (%)**	***P***
ctDNA mutations detected before the start of FOLFIRINOX				
Any ctDNA mutation	4 (22.2)	5 (31.3)	8 (57.1)	0.112
KRAS	3 (16.7)	3 (18.8)	6 (42.9)	0.184
TP53	2 (11.1)	2 (12.5)	4 (28.6)	0.363
PIK3CA	2 (11.1)	0 (0)	0 (0)	0.176
ctDNA mutations detected after one cycle of FOLFIRINOX				
Any ctDNA mutation	3 (16.7)	6 (37.5)	1 (7.1)	0.107
KRAS	2 (11.1)	3 (18.8)	0 (0)	0.243
TP53	2 (11.1)	2 (12.5)	0 (0)	0.403
PIK3CA	2 (11.1)	0 (0)	0 (0)	0.176
Germline variants				
TP53 Pro72Arg				
Pro/Pro	1 (5.6	2 (12.5)	0 (0)	0.579
Pro/Arg	5 (27.8)	4 (25.0)	6 (42.9)	
Arg/Arg	12 (66.7)	10 (62.5)	8 (57.1)	
Pro/Pro + Pro/Arg	6 (33.3)	6 (37.5)	6 (42.9)	0.859
Arg/Arg	12 (66.7)	10 (62.5)	8 (57.1)	
KDR Gln472His				
Gln/Gln	10 (55.6)	9 (56.3)	9 (64.3)	0.983
Gln/His	6 (33.3)	5 (31.3)	4 (28.6)	
His/His	2 (11.1)	2 (12.5)	1 (7.1)	
KIT Met541Leu				
Met/Met	14 (77.8)	13 (81.3)	11 (78.6)	0.886
Met/Leu	3 (16.7)	3 (18.8)	2 (14.3)	
Leu/Leu	1 (5.6)	0 (0)	1 (7.1)	
ERBB2 Ile625Val				
Ile/Ile	10 (55.6)	8 (50.0)	9 (64.3)	0.719
Ile/Val	6 (33.3)	7 (43.8)	5 (35.7)	
Val/Val	2 (11.1)	1 (6.3)	0 (0)	
PIK3CA Ile391Met				
Ile/Ile	18 (100)	14 (87.5)	11 (78.6)	0.142
Ile/Met	0 (0)	1 (6.3)	3 (21.4)	
Met/Met	0 (0)	1 (6.3)	0 (0)	

Arg = arginine, Gln = glutamine, His = histidine, Ile = isoleucine, Leu = leucine, Met = methionine, Pro = proline, Val = valine.



Supplementary Figure 1. Circulating cell-free DNA (ccfDNA) concentration dynamics in pancreatic cancer patients during one cycle of FOLFIRINOX.

Concentrations (conc.) of ccfDNA were corrected for short fragment sized DNA, based on Alu115 primer RT-qPCR measurements. Data are presented as medians with interquartile ranges. Wilcoxon signed rank tests were used to compare ccfDNA concentrations before and after chemotherapy, Mann-Whitney U tests to compare DNA concentrations between disease control and progressive disease patients (ns = not significant, * = $P<0.05$, ** = $P<0.01$).



Supplementary Figure 2. Variant allele frequencies (VAF) of circulating tumor DNA (ctDNA) mutations detected per patient sample.

21/48 patients had at least one detectable ctDNA mutation in one of the samples (before or after one cycle of chemotherapy).

B = before start of FOLFIRINOX, A = after one cycle of FOLFIRINOX, fs = frame shift, * = stop codon. Color legend: green is lowest, yellow is median, red is highest VAF (range: 1.02-7.56%).

Chapter 6

Serum miR-373-3p and miR-194-5p are associated with early tumor progression during FOLFIRINOX treatment in pancreatic cancer patients: a prospective multicenter study

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ABSTRACT

In this study, we explored the predictive value of serum microRNA (miRNA) expression for early tumor progression during FOLFIRINOX chemotherapy and its association with overall survival (OS) in patients with pancreatic ductal adenocarcinoma (PDAC). A total of 132 PDAC patients of all disease stages was included in this study, of whom 25% showed progressive disease during FOLFIRINOX according to the RECIST criteria. MiRNA expression was analyzed in serum collected before start and after one cycle of chemotherapy. In the discovery cohort (*n*=12), a 352-miRNA RT-qPCR panel was used. In the validation cohorts (total *n*=120), miRNA expression was detected using individual RT-qPCR miRNA primers. Before start of FOLFIRINOX, serum miR-373-3p expression was higher in patients with progressive disease compared to patients with disease control after FOLFIRINOX (Log2 fold difference (FD) 0.88, *P*=0.006). MiR-194-5p expression after one cycle of FOLFIRINOX was lower in patients with progressive disease (Log2 FD -0.29, *P*=0.044). Both miRNAs were predictors of early tumor progression in a multivariable model including disease stage and baseline CA19-9 level (miR-373-3p odds ratio (OR) 3.99, 95% CI 1.10-14.49; miR-194-5p OR 0.91, 95% CI 0.83-0.99). MiR-373-3p and miR-194-5p did not show an association with OS after adjustment for disease stage, baseline CA19-9, and chemotherapy response. In conclusion, high serum miR-373-3p before start and low serum miR-194-5p after one cycle are associated with early tumor progression during FOLFIRINOX.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer associated with poor prognosis.[1] Its incidence is rising and predictions show that PDAC could become the second leading cause of cancer-related deaths by 2030.[2] Unfortunately, there has been only a slight improvement in the treatment of PDAC in the last decennium. Most patients will receive chemotherapy, of which FOLFIRINOX, a combination of fluorouracil, leucovorin, irinotecan and oxaliplatin, is one of the most effective and most commonly used regimens. First-line FOLFIRINOX prolongs survival of advanced PDAC patients[3, 4] as well as survival in patients with resectable or borderline resectable disease when administered as adjuvant treatment.[5] Although FOLFIRINOX will stabilize the disease in most patients, median overall survival (OS) is still only 11 months in patients with metastatic PDAC.[4] Furthermore, patients often experience severe chemotherapy-induced toxicity.[3-6]

Chemotherapy resistance is one of the main reasons for the lack of survival benefit. In PDAC, both intrinsic and acquired mechanisms play a role in chemotherapy resistance.[7] Intrinsic chemotherapy resistance is caused by the dysregulation of the tumor microenvironment: PDAC tumors are surrounded by a dense stromal layer, are poorly vascularized, and rich in tumor-promoting, immunosuppressive components.[7-9] Acquired chemotherapy resistance is the result of selection of cancer cells with mechanisms leading to insensitivity to chemotherapy. These include, for example, insensitivity to chemotherapy-induced apoptosis, increased DNA repair mechanisms, dysregulation of the cell cycle, and multidrug resistance caused by increased expression of cell membrane transporters that eliminate chemotherapeutic drugs from the cell.[7, 9, 10]

Many of these chemotherapy resistance processes are regulated by non-coding RNAs.[10, 11] These microRNAs (miRNAs) are important post-transcriptional regulators of gene expression by modulation of target messenger RNA (mRNA). A single miRNA can regulate multiple genes, and one gene can be influenced by many different miRNAs. Therefore, miRNAs can alter most cellular processes, including processes initiating chemotherapy resistance.[10, 11]

Dysregulation of miRNA expression could potentially predict chemotherapy resistance and guide patient selection for chemotherapy-based treatment, such as FOLFIRINOX.[10, 12] In this study, we measured circulating miRNA expression in serum before and after one cycle of treatment in PDAC patients with disease control and progressive disease after FOLFIRINOX. MiRNA expression was measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Additionally, we explored the predictive value of serum miRNAs for early disease progression and their prognostic value for overall survival (OS).

MATERIALS AND METHODS

This article was written according to the Reporting recommendations for tumor marker prognostic studies (REMARK) guidelines.[13]

Patient selection

All patients, initially treated with FOLFIRINOX, were selected from two multicenter, prospective trials in the Netherlands. Patients with resectable or borderline resectable PDAC participated in the randomized clinical trial PREOPANC-2 (Dutch trial register NL7094) comparing neoadjuvant FOLFIRINOX to neoadjuvant gemcitabine-based chemoradiotherapy, followed by surgical resection of the primary tumor if applicable. Patients with locally advanced and metastatic PDAC were selected from the prospective cohort study iKnowIT (Dutch trial register NL7522) investigating the predictive value of circulating biomarkers. The trials were approved by the ethics committees of all participating hospitals: Erasmus MC (ethics committee reference number MEC-2018-087 and MEC-2018-004), Amsterdam UMC (2018_196 and 2018_138), Leiden University Medical Center (L18.070 and L18.053), Isala hospital, Zwolle (180606), Reinier de Graaf Gasthuis, Delft (SK/CS 19-119), Jeroen Bosch hospital, Den Bosch (2018.07.17.01), Maasstad hospital, Rotterdam (L2018053 and L2018095), Onze Lieve Vrouwe Gasthuis, Amsterdam (WO 18.118), and Medisch Spectrum Twente, Enschede (H18-081).

Due to the explorative character of this study, no formal sample size calculation was performed. Patients were selected based on the availability of serum samples and treatment response outcome.

After histopathological confirmation of the primary tumor and/or metastases, patients from all PDAC disease stages received initial treatment with FOLFIRINOX between February 2018 and November 2020. Patients received a maximum of 12 cycles. Exclusion criteria for patient selection were age under 18 years, co-treatment with other chemotherapeutics, and previous treatment with FOLFIRINOX. A staging CT scan was performed maximum four weeks prior to start of chemotherapy. A CT scan to evaluate the tumor response to treatment was performed after every fourth cycle of FOLFIRINOX, or earlier if patients showed clinical signs of tumor progression, according to the Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria[14] as part of standard clinical practice. Final treatment response was defined as the treatment response measured on the CT scan immediately after the last cycle of FOLFIRINOX. In patients with progressive disease, FOLFIRINOX was discontinued. Disease control was defined as stable disease, partial, or complete response. Patients with disease control continued with FOLFIRINOX for a maximum of 12 cycles. Patient characteristics, such as age, sex, stage of disease, laboratory results, CT scan evaluations, and follow-up data were retrieved from medical records by a medical doctor. Follow-up ended upon the death of the patient.

Sample collection

Peripheral venous blood samples were collected before the start of FOLFIRINOX and two weeks after the first cycle of FOLFIRINOX. Blood was collected in 10 mL serum tubes with clot activator of silica particles (Becton Dickinson, Franklin Lakes, NJ, USA). Within two hours after collection, blood samples were centrifuged for ten minutes at 2000 g, and serum was stored at -80 °C until further use.

Serum miRNA isolation and quantitation

MiRNAs were analyzed in three cohorts: a discovery cohort, validation cohort 1, and validation cohort 2. Only differentially expressed serum miRNAs between patients with disease control and patients with progressive disease with a fold difference (FD) of ≤0.33 or ≥3 (corresponding to a Log2 FD of ≤-1.59 or ≥1.59) and $P<0.05$, that were detectable within the raw threshold cycle (Ct) value limits in all twelve patients from the discovery cohort, were selected for validation cohort 1. MiRNAs that remained statistically significantly up- or downregulated were selected for validation cohort 2. MiRNA selection in the different cohorts is visualized in Figure 1.

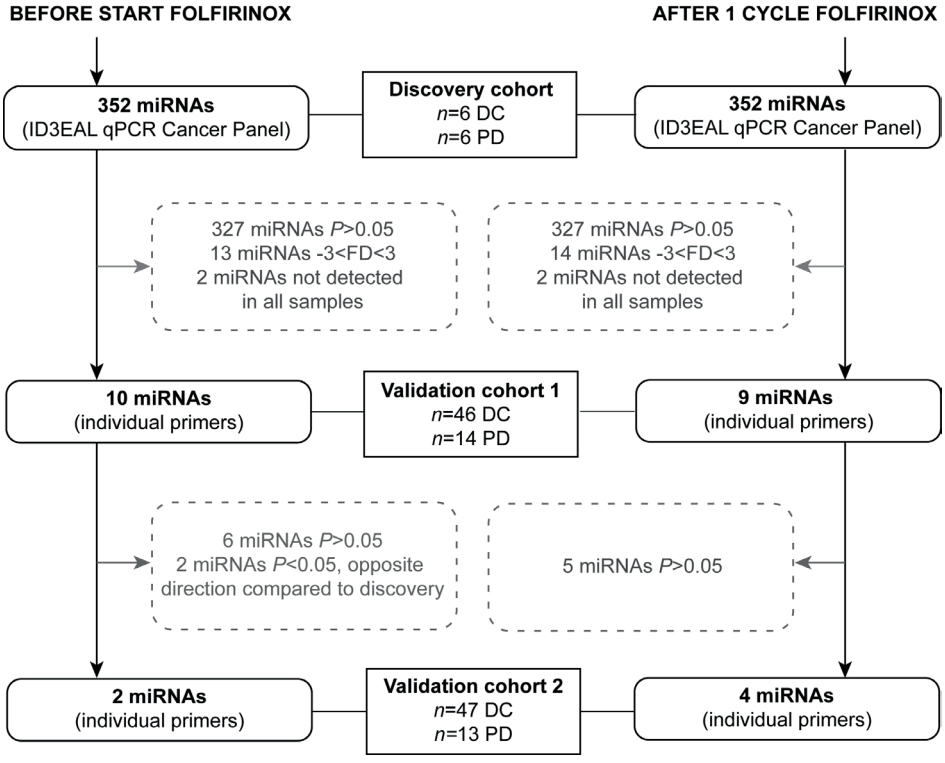

Figure 1. Flowchart of the selection of microRNAs from the discovery panel for the two validation cohorts.
DC = disease control, FD = fold difference, PD = progressive disease.

RNA was isolated from 2 x 200 µL or 200 µL serum using the miRNeasy serum/plasma miRNA Isolation Kit (Qiagen, Hilden, Germany) for the discovery cohort and validation cohorts, respectively. In the validation cohorts, three proprietary pre-mixed spike-in ~20 nucleotide control RNAs (MiRXES, Singapore) with sequences distinct from annotated mature human miRNAs (miRbase version 21) were added to the lysis buffer prior to the serum miRNA isolation according to the manufacturer's instructions, in order to evaluate RNA isolation efficiency.

In the discovery cohort, serum miRNAs were reverse transcribed (RT) using ID3EAL miRNA-specific oligo's and RT spike-in RNA (MiRXES, Singapore) in a multiplex reaction per manufacturer's instruction. Complementary DNA (cDNA) was stored at -20°C up to two weeks and thawed only once. cDNA was added to the ID3EAL miRNA qPCR Master Mix, containing buffer, polymerase and the passive reference dye ROX, and transferred to pre-loaded ID3EAL 384 Target Assay Panel plates (MiRXES, Singapore), including 352 individual quantitative polymerase chain reaction (qPCR) primers, 16 RT spike-ins and 16 inter-plate controls. PCR amplification was performed with the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Raw Ct values were determined using the 7500 Software (version 2.3; Applied Biosystems, Foster City, CA, USA).

In the two validation cohorts, selected serum miRNAs were measured with ID3EAL RT-qPCR after reverse transcription, using individual miRNA primers (MiRXES, Singapore). Raw Ct values were determined using the 7500 Software (version 2.3; Applied Biosystems, Foster City, CA, USA).

In the discovery cohort, raw Ct values were normalized using reference miRNAs, spike-ins, and inter-plate calibrators, using an algorithm incorporated in the Cancer Panel Analysis Template (version 1.9, MiRXES, Singapore), provided by the manufacturer. The two miRNAs with the most stable expression among all samples (miR-26a-5p and miR-30b-5p) were selected as reference miRNAs, for both the discovery and validation cohorts, using NormFinder software for Excel (version 0.953; MOMA, Aarhus University Hospital, Denmark). The cutoff values for detection of raw Ct values were 9-33 cycles, based on the manufacturer's recommendations. In the validation cohorts, raw Ct values were normalized using the same two reference miRNAs (miR-26a-5p and miR-30b-5p). Data from the validation cohorts were normalized and analyzed using the online Thermo Fisher Connect Platform (Thermo Fisher Scientific, Waltham, MA, USA).

In situ hybridization (ISH) of pancreatic cancer tissue

Diagnostic biopsies of PDAC and PDAC liver metastases were collected at the Erasmus MC for clinical pathology evaluation. Stored formalin-fixed paraffin-embedded (FFPE)

tissue blocks were analyzed for clinical histopathological diagnosis and residual material was used for biomarker analysis. Four micrometer thick tissue sections were processed in the Discovery Ultra instrument (Ventana Medical Systems, Oro Valley, AZ, USA) with the automated Discovery Universal protocol. In brief, after deparaffinization and heat-induced antigen retrieval with CC1 (#950-124, Ventana) for 16 minutes at 100 °C followed by ISH protease 2 (#780-4148, Ventana) for 4 minutes at 37 °C, peroxidase inhibitor CM (#760-159, Ventana) was added for 8 minutes followed by adding 20 nM of 3' and 5' – DIG labeled miRCURY LNA miRNA miR-373-3p detection probes (Qiagen, Hilden, Germany) for 8 minutes at 84 °C. Hybridization at 55 °C for 1 hour was followed by wash steps with 1xSSC (#950-110, Ventana). Detection with anti-DIG HRP (#760-4822, Ventana) and Disc Amp BF (#760-226, Ventana) was followed by visualization with DAB (#760-159, Ventana). The tissues were counterstained with Hematoxylin II (Ventana). Adjacent tissue sections were stained with hematoxylin and eosin (HE). The slides were scanned using the Nanozoomer 2.0-HT slide imager (Hamamatsu Photonics, Hamamatsu City, Japan).

Statistical analysis

Patient characteristics were compared between the different cohorts with Kruskall Wallis tests for continuous data, including age, number of FOLFIRINOX cycles, and baseline CA19-9 levels, and with Chi-squared tests for categorical data: sex, stage of disease, and RECIST response outcome.

MiRNA expression was analyzed in three ways: differences in miRNA expression before start of FOLFIRINOX, miRNA expression after one cycle of FOLFIRINOX, and differences in miRNA expression change over time between patients with disease control and patients with progressive disease.

MiRNA expression relative to the reference miRNAs was calculated with the delta Ct method; expression = $2^{-(\text{Ct miRNA of interest} - \text{average Ct reference miRNAs})}$. The mean miRNA expression was compared between patients with disease control and patients with progressive disease using a two-tailed t-test. The disease control patient group was selected as the reference group to calculate fold differences between disease control and progressive disease patients. In addition, fold of change (FOC) in miRNA expression within groups after one cycle of FOLFIRINOX was tested with paired t-tests. Statistical significance of FOC over time between disease control and progressive disease patients was compared with two-tailed t-tests. Only miRNAs that showed opposite directions of the FOC in disease control and progressive disease patients, meaning upregulated in one group, downregulated in the other, analyzed with paired t-tests, were found clinically significant and therefore selected for further analyses. Differences in miRNA expression between

stages of disease was tested with one-way ANOVA. Correlations between expression of different miRNAs were tested with Pearson's correlation.

Univariable and multivariable binary logistic regression was performed to analyze the predictive value of relative miRNA expression and known predictive tumor characteristics: stage of disease, and baseline serum CA19-9 level. Variables with $P<0.10$ were selected for multivariable analysis.

Overall survival (OS) was calculated as the time between the start of FOLFIRINOX and death. The prognostic value of circulating miRNA expression was tested with univariable and multivariable Cox regression analysis, including known prognostic factors: age, stage of disease, chemotherapy response, and baseline serum CA19-9 level.

Statistical analyses were performed with the online Thermo Fisher Connect Platform (Thermo Fisher Scientific, Waltham, MA, USA) and SPSS Statistics for Windows (version 25.0; IBM, Armonk, NY, USA). *P*-values <0.05 were considered statistically significant.

RESULTS

Patient characteristics

For the three cohorts combined, the discovery cohort (n=12), validation cohort 1 (n=60), and validation cohort 2 (n=60), a total of 132 patients were selected for circulating miRNA analysis. Patient characteristics are presented in Table 1. Almost half of the patients (47.0%) presented with resectable or borderline resectable PDAC, 34.1% with locally advanced pancreatic cancer (LAPC), and 18.9% with metastatic PDAC. The percentage of patients with resectable or borderline resectable disease was higher in the validation cohorts compared to the discovery cohort (P=0.016), due to the low availability of samples at the beginning of the PREOPANC-2 trial. There were no other significant differences in patient characteristics between cohorts.

Serum miRNA expression in the discovery cohort

A step-wise approach, shown in Figure 1, was used to select serum miRNAs of interest from an exploratory screening panel. In the discovery cohort, consisting of six disease control patients and six patients with progressive disease after FOLFIRINOX treatment, 352 miRNAs were analyzed. The miRNAs miR-26a-5p and miR-30b-5p combined showed the best stability value based on 24 discovery samples (twelve before start of FOLFIRINOX and twelve after one cycle of FOLFIRINOX) and were selected as reference miRNAs for all cohorts.

Table 1. Patient characteristics.

	Discovery cohort (*n*=12)	**Validation cohort 1 (*n*=60)**	**Validation cohort 2 (*n*=60)**	***P***	**Total cohort (*n*=132)**
Age (years), median (range)	64 (49-78)	66 (41-81)	62 (49-79)	0.601	64 (41-81)
Sex, male (%)	7 (58.3)	34 (56.7)	36 (60.0)	0.934	77 (58.3)
Stage of disease (%)					
(Borderline) resectable	2 (16.7)	29 (48.3)	31 (51.7)	0.016	62 (47.0)
Locally advanced	4 (33.3)	18 (30.0)	23 (38.3)		45 (34.1)
Metastatic	6 (50.0)	13 (21.7)	6 (10.0)		25 (18.9)
Cycles of FOLFIRINOX received, median (range)	4 (2-12)	8 (2-12)	8 (1-12)	0.087	8 (1-12)
Baseline CA19-9 (kU/L), median (IQR)	410 (74.5-9341.0)	147.5 (51.8-910.3)	216.0 (51.0-845.0)	0.315	190.0 (51.0-1050.0)
RECIST response outcome after FOLFIRINOX[a] (%)					
Disease control	6 (50.0)	46 (76.7)	47 (78.3)	0.108	99 (75.0)
Progressive disease	6 (50.0)	14 (23.3)	13 (21.7)		33 (25.0)

[a] According to the RECIST 1.1 criteria. CA19-9 = carbohydrate antigen 19-9, IQR = interquartile range. *P*-values are calculated by Kruskal Wallis tests (continuous data) or Chi-squared tests (categorical data).

Before the start of FOLFIRINOX, ten miRNAs from the 352-miRNA panel showed a statistically significant fold difference between patients with disease control and patients with progressive disease, as shown in Table 2. From these ten, four miRNAs were upregulated, and six were downregulated in patients with progressive disease compared to patients with disease control. After one cycle of FOLFIRINOX, nine miRNAs showed a significant fold difference. All nine miRNAs were downregulated in patients with progressive disease compared to patients with disease control after FOLFIRINOX. The miRNAs let-7g-5p, miR-194-5p, miR-30a-5p were both before and after one cycle of FOLFIRINOX downregulated in patients with progressive disease. MiR-10a-5p and let-7f-5p showed opposite changes in expression after one cycle of FOLFIRINOX between patients with disease control and patients with progressive disease. In patients with disease control, miR-10a-5p showed an increase (Log2 fold of change (FOC) 0.78) in expression after one cycle of chemotherapy, while a decrease (Log2 FOC -0.22) in progressive disease patients (*P*=0.006, Figure 2A). Let-7f-5p showed a decrease (Log2 FOC -0.80) after one cycle of chemotherapy in disease control and increase (Log2 FOC 0.52) in progressive disease patients (*P*=0.046, Figure 2B).

A total of eighteen differently expressed miRNAs from the discovery panel, before the start of FOLFIRINOX, after one cycle of FOLFIRINOX, or both, and those with different expression patterns over time were selected for validation cohort 1.

Table 2. Differences in serum miRNA expression between pancreatic cancer patients with disease control and patients with progressive disease after FOLFIRINOX.

	Discovery cohort (*n*=6 DC, *n*=6 PD)		Validation cohort 1 (*n*=46 DC, *n*=14 PD)		Validation cohort 2 (*n*=47 DC, *n*=13 PD)		Total validation cohort (*n*=93 DC, *n*=27 PD)	
miRNA	**Log2 FD***	***P***	**Log2 FD***	***P***	**Log2 FD***	***P***	**Log2 FD***	***P***
Before start of FOLFIRINOX								
hsa-let-7g-5p[a]	-2.34	0.041	0.27	0.189				
hsa-miR-126-3p	-1.75	0.004	0.08	0.568				
hsa-miR-1290	1.78	0.039	0.28	0.451				
hsa-miR-17-3p	2.11	0.038	0.49	0.048	0.11	0.752	0.36	0.134
hsa-miR-194-5p[a]	-2.44	0.015	0.57	0.112				
hsa-miR-199a-5p	-1.64	0.014	-0.34	0.254				
hsa-miR-200c-3p	3.52	0.032	0.48	0.185				
hsa-miR-30a-5p[a]	-2.64	0.041	0.41	0.007				
hsa-miR-373-3p	8.37	<0.001	0.84	0.110	0.92	0.007	0.88	0.006
hsa-miR-629-5p	-3.56	0.048	0.58	0.015				
After one cycle of FOLFIRINOX								
hsa-let-7g-5p[a]	-1,66	0.020	-0.09	0.570				
hsa-miR-18a-5p	-1,83	0.007	-0.32	0.027	0.56	0.016	0.13	0.361
hsa-miR-19a-3p	-1,72	0.049	-0.05	0.793				
hsa-miR-194-5p[a]	-2,25	0.017	-0.50	0.026	-0.15	0.421	-0.29	0.044
hsa-miR-24-3p	-3,92	0.036	-0.78	0.024	0.53	0.073	-0.08	0.715
hsa-miR-27a-3p	-2,22	0.041	-0.95	0.008	0.45	0.208	-0.20	0.459
hsa-miR-30a-5p[a]	-1,78	0.020	-0.16	0.235				
hsa-miR-30d-5p	-3,35	<0.001	-0.15	0.205				
hsa-miR-92b-3p	-1,94	0.049	0.16	0.563				

[a] MicroRNAs were selected for validation in both samples before start of FOLFIRINOX as well as samples after one cycle of FOLFIRINOX. DC = disease control, FD = fold difference, hsa = homo sapiens (human), miR/miRNA = microRNA, PD = progressive disease. *The disease control patient group was set as the reference group. *P*-values by t-tests.

Serum miRNA expression in the validation cohorts

The results of both validation cohorts are presented in Table 2. In validation cohort 1, only miR-17-3p remained statistically significantly expressed between disease control and progressive patients before the start of FOLFIRINOX (Log2 FD 0.49, *P*=0.048). Two miRNAs, miR-30a-5p and miR-629-5p, also showed a significantly higher expression in progressive disease patients in validation cohort 1. Contrarily, these same miRNAs were downregulated in the discovery cohort and therefore not selected for further evaluation. After one cycle of FOLFIRINOX, miR-18a-5p (Log2 FD -0.32, *P*=0.027), miR-194-5p (Log2 FD -0.50, *P*=0.026), miR-24-3p (Log2 FD -0.78, *P*=0.024), and miR-27a-3p (Log2 FD -0.95, *P*=0.008) remained significantly downregulated in patients with progressive disease compared to patients with disease control. These five statistically significant miRNAs

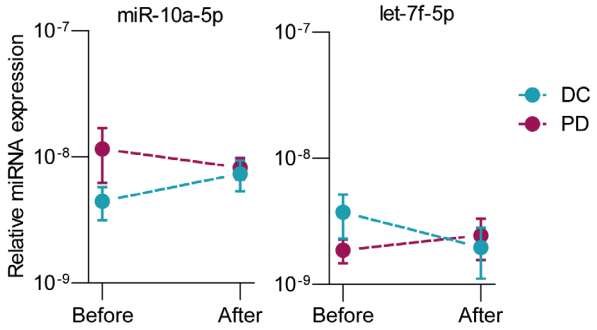

Figure 2. Changes in serum miRNA expression over time measured before start of FOLFIRINOX and after one cycle of FOLFIRINOX with opposite directions between patients with disease control (DC, *n*=6) and progressive disease (PD, *n*=6) in the discovery cohort.
Data is presented as average FD, relative to the expression of the two reference miRNAs, with standard deviations. *P*-values were calculated with paired t-tests.

(one before start of FOLFIRINOX, four after one cycle of FOLFIRINOX) were selected for additional validation in validation cohort 2. In addition, miR-373-3p was also selected for validation cohort 2. miR-373-3p was the miRNA with the highest fold difference in the discovery cohort and therefore a promising predictive biomarker. Also, it has been reported as an important cancer miRNA in literature. The Ct values of miR-373-3p were significantly lower in validation cohort 1, suggesting a technical difference between the 352-miRNA discovery panel and the individual primers.

In validation cohort 2, miR-373-3p (Log2 FD 0.92. *P*=0.007) before start of FOLFIRINOX, and miR-18a-5p (Log2 FD 0.56, *P*=0.016) after one cycle of FOLFIRINOX showed significant FD between progressive disease compared to patients with disease control (Table 2). However, while miR-18a-5p was upregulated in validation cohort 2, it was downregulated in validation cohort 1. Due to this discrepancy, we did not further investigate miR-18a-5p.

When combining the results of both validation cohorts, miR-373-3p (Log2 FD 0.88, *P*=0.006) before the start of FOLFIRINOX, and miR-194-5p (Log2 FD -0.29, *P*=0.044) after one cycle of FOLFIRINOX remained significantly differently expressed between patients with progressive disease and patients with disease control (Table 2).

In a multivariable model, miR-373-3p expression before therapy (OR 3.99, 95% CI 1.10-14.49, *P*=0.035) and miR-194-5p expression after one cycle of FOLFIRINOX (OR 0.91, 95% CI 0.83-0.99, *P*=0.030) remained significant predictive factors of early tumor progression during FOLFIRINOX (Table 3). Expression of miR-373-3p and expression of miR-194-5p was not correlated (Pearson's r=0.032, *P*=0.742).

Table 3. Univariable and multivariable binary logistic regression model for the prediction of early tumor progression during FOLFIRINOX.

	Univariable		Multivariable	
Variable	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Stage of disease				
Resectable	Ref			
LAPC	1.31 (0.58-2.95)	0.521		
Metastatic	1.87 (0.65-5.35)	0.245		
CA19-9 at baseline (per 100 kU/L)	1.00 (1.00-1.01)	0.475		
miR-17-3p relative expression over reference miRNAs (per $1x10^{-2}$ increase)[a]	1.39 (0.91-2.13)	0.125		
miR-373-3p relative expression over reference miRNAs (per $1x10^{-2}$ increase)[a]	2.62 (0.90-7.63)	0.078	3.99 (1.10-14.49)	0.035
miR-194-5p relative expression over reference miRNAs (per $1x10^{-2}$ increase)[b]	0.94 (0.87-1.00)	0.065	0.91 (0.83-0.99)	0.030

[a] In samples before start of FOLFIRINOX, [b] in samples after one cycle of FOLFIRINOX. CA19-9 = carbohydrate antigen 19-9, CI = confidence interval, OR = odds ratio, miR/miRNA = microRNA, Ref = reference.

Serum miRNA expression between disease stages

No difference in serum miRNA expression was found between stages of disease for any of the miRNAs from the validation cohorts, except for miR-17-3p (Supplementary Table 1). MiR-17-3p was overexpressed in resectable disease patients with early tumor progression during FOLFIRINOX compared to resectable patients with disease control (Log2 FD 0.58, *P*=0.040, Supplementary Table 2). This difference in miR-17-3p expression was not found in patients with LAPC or metastatic disease.

Serum miRNA expression and overall survival

The median follow-up time was 14.0 months for patients alive at last follow-up. The median OS for the total cohort of PDAC patients was 11.7 months. In univariable analyses, miR-373-3p and miR-194-5p expression were not associated with OS. Serum miR-17-3p expression before start of FOLFIRINOX was a prognostic factor for OS (HR 1.30, 95% CI 1.02-1.65, *P*=0.032), as shown in Table 4. However, in multivariable analysis, after adjustment for stage of disease, baseline CA19-9 level, and RECIST chemotherapy response outcome, miR-17-3p expression did not remain a significant predictor of OS (HR 1.18, 95% CI 0.92-1.52, *P*=0.192).

Tissue miR-373-3p expression

To assess the origin of serum miR-373-3p expression, treatment-naïve PDAC and PDAC metastasis tissue biopsies were analyzed by in situ hybridization (ISH) with miR-373-3p probes. Positive and negative control tissue staining with U6 and scramble miRNA

Table 4. Univariable and multivariable Cox proportional hazards model for overall survival (OS) after FOLFIRINOX.

	Univariable		Multivariable	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age (per year)	1.01 (0.99-1.04)	0.226		
Stage of disease				
Resectable	Ref		Ref	
LAPC	1.00 (0.66-1.54)	0.985	1.40 (0.72-2.71)	0.316
Metastatic	2.16 (1.36-3.43)	0.001	2.51 (1.21-5.23)	0.014
CA19-9 at baseline (per 100 kU/L)	1.00 (1.00-1.01)	0.009	1.00 (1.00-1.01)	0.116
RECIST response outcome				
Disease control	Ref		Ref	
Progressive disease	3.85 (2.58-5.73)	<0.001	4.64 (2.48-8.68)	<0.001
miR-17-3p relative expression over reference miRNAs (per $1x10^{-2}$ increase)[a]	1.30 (1.02-1.65)	0.032	1.18 (0.92-1.52)	0.192
miR-373-3p relative expression over reference miRNAs (per $1x10^{-2}$ increase)[a]	1.15 (0.96-1.38)	0.141		
miR-194-5p relative expression over reference miRNAs (per $1x10^{-2}$ increase)[b]	0.96 (0.90-1.02)	0.145		

[a] In samples before start of FOLFIRINOX, [b] in samples after one cycle of FOLFIRINOX. LAPC = locally advanced pancreatic cancer, CA19-9 = carbohydrate antigen 19-9, CI = confidence interval, HR = hazard ratio, miR/miRNA = microRNA, Ref = reference.

probes is visualized in Supplementary Figure 1. The miR-373-3p expression in healthy tissues is shown in Supplementary Figure 2. MiR-373-3p is expressed by normal endothelium, colon epithelial cells, hepatocytes, renal tubular cells, neurons, and tonsillar B cell lymphoid follicles. Lung epithelium and T cells do not express detectable levels of miR-373-3p. In normal pancreatic tissue, miR-373-3p is expressed in acinar cells only, pancreatic ductal cells do not express miR-373-3p (Supplementary Figure 1). However, pancreatic ductal adenocarcinoma cells, both in primary PDAC tissues as well as PDAC liver metastases, express high levels of miR-373-3p (Figure 3).

Because tumor samples after one cycle of treatment were not available, miR-194-5p was not further investigated by ISH, since this miRNA showed predictive value during FOLFIRINOX treatment instead of before start of treatment.

DISCUSSION

In this multicenter, prospective study we found that PDAC patients with progressive disease compared to patients with disease control showed higher expression of serum miR-373-3p before start of FOLFIRINOX and lower expression of serum miR-194-5p after

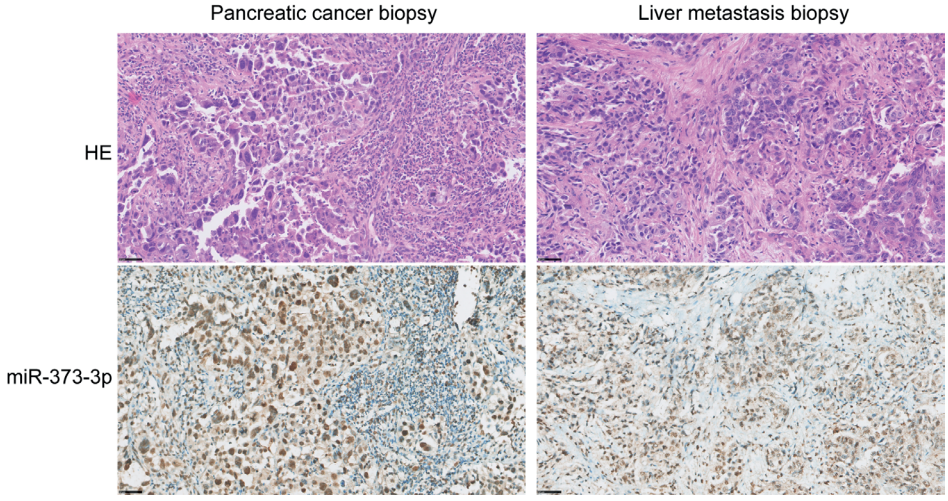

Figure 3. In situ hybridization of treatment-naïve pancreatic ductal adenocarcinoma (PDAC) and PDAC metastasis biopsies for miR-373-3p.
Tissue sections from a primary PDAC tumor and from a PDAC liver metastasis (before FOLFIRINOX) are stained with hematoxylin and eosin (HE) and with miR-373-3p, visualized with DAB. Scalebar = 100 µm.

one cycle of FOLFIRINOX. In multivariable logistic regression, both miRNAs were significant predictors of early tumor progression during FOLFIRINOX. Expression of serum miR-373-3p and miR-194-5p was not associated with OS.

Studies investigating circulating miRNAs in PDAC patients undergoing chemotherapy are scarce and currently no miRNA biomarker has been validated for clinical use. Several studies have been published on the diagnostic[15-17] and prognostic value[17-20] of circulating miRNAs. The present study is one of the firsts on circulating miRNAs and their value to predict treatment outcome in patients with PDAC. A previous study by Meijer *et al.* showed that patients with early progression after completion of FOLFIRINOX treatment, overexpress plasma miR-181a-5p after treatment compared to patients with longer progression-free survival, as measured with RT-qPCR.[12] In our discovery cohort, miR-181a-5p did not show a difference in expression between patients with disease control and patients with progressive disease during FOLFIRINOX. However, an important difference between the two studies is the time point of blood collection and miRNA measurement. Meijer *et al*. collected plasma samples from 54 advanced PDAC patients after 5-6 cycles of FOLFIRINOX,[12] while we collected blood for serum miRNA expression analysis after only one cycle of FOLFIRINOX. The more cycles of chemotherapy patients receive, the more evident the differences in biological (non)response to treatment will become. In contrast, we measured miRNA expression before start and in an early phase of treatment; a predictive biomarker before chemotherapy or after one cycle can better guide subsequent treatment.

Other miRNAs have been described for their role in chemotherapy response.[21, 22] For example miR-200b, miR-200c, and miR-21 were often found to be involved in 5-FU resistance.[21] Unfortunately, these findings on miRNAs influencing chemotherapy response are based on research performed in cell lines,[21] which do not resemble pancreatic tumor tissue from patients. Cell lines might undergo genotypic and phenotypic transformation and are not exposed to cancer-associated environmental components, including stromal and immunological factors.[23] Especially in miRNA research the interaction with tumor stroma is important, since a large part of circulating miRNAs originate from endothelium and immune cells and circulating miRNAs often target messenger RNAs involved in immune responses.[24, 25]

In this study, we found serum miR-373-3p and miR-194-5p to be associated with early tumor progression during FOLFIRINOX. Two studies both showed downregulation of miR-373-3p in PDAC patients compared to healthy individuals in tissue[26] and serum samples.[27] Lower expression was also associated with poor prognostic clinical features and OS.[27] These findings are inconsistent with the results of the present study in which upregulation of miR-373-3p was associated with early tumor progression. The difference in miR-373 expression could lie in differences between the patient populations. The description of patient characteristics in these two studies, however, is insufficient to allow for comparison. Our ISH results show that miR-373-3p is expressed by normal acinar cells, and highly expressed by PDAC cells, whereas no expression was detected in the tumor stroma, which is the largest component of PDAC tumors. RNA from homogenized tumor tissue does not allow for cell-specific miRNA measurements, which might explain differences between our findings and those described in the literature. On the other hand, miR-373-3p is known to act in an ambiguous way; in some cancers this miRNA has been described as a tumor suppressor (e.g., in perihilar cholangiocarcinoma), while in other cancers miR-373 shows tumor promoting properties (e.g., in hepatocellular carcinoma and breast cancer).[28] Many genes have been identified as targets of miR-373-3p.[28] One of the most interesting functions of miR-373 in the light of PDAC, is that this miRNA cooperates with oncogenic RAS to overcome the need for P53 loss to achieve cancer cell proliferation, which was demonstrated in testicular germ-cell tumors. The wild-type (WT) *TP53* gene, and its P53 protein, is a tumor suppressor inducing cellular senescence. Normally, P53 will have an anti-proliferative response to oncogenic RAS-induced tumorigenesis. However, expression of miR-373 inactivates P53 and therefore the senescence mechanism is bypassed.[28, 29] We found high levels of miR-373-3p in PDAC cells, in contrast to normal pancreatic ductal cells that do not show miR-373-3p tissue expression with ISH. This, together with the fact that serum miR-373-3p levels are already higher before the start of treatment in patients with disease progression upon

FOLFIRINOX, also suggests that miR-373-3p is a PDAC cell-intrinsic malignant factor and not an, for example, immunologic miRNA in response to PDAC.

KRAS and *TP53* are the most frequently mutated genes in PDAC.[30] Investigating if miR-373 levels differ between PDAC patients with WT *TP53* and patients with *TP53* mutations would be an interesting next step. A relative upregulation of miR-373 might only be detected in patients with mutated *KRAS* in combination with WT *TP53*. Unfortunately, the mutational status is unknown for most patients included in our patient cohort.

To our knowledge, miR-194-5p has been reported in PDAC patients only once. This miRNA was overexpressed in PDAC tissue and serum samples compared to healthy controls and ectopic expression of miR-194-5p in PDAC cell lines promoted cell proliferation and migration.[31] This is in contrast to our findings. However, in multiple different cancer experiments, including gastric cancer, lung cancer, and nasopharyngeal cancer, it is shown that miR-194 suppresses cancer cell proliferation, which is in line with our results.[32-34] Also, in osteosarcoma and colorectal cancer, low serum miR-194 was associated with poor prognosis, comparable to our findings.[35, 36]

The bidirectional, somewhat ambiguous results in the literature is an important limitation of miRNA research in general. MiRNAs target many mRNAs and proving miRNA involvement in different cellular pathways is challenging. The function of miRNAs differs between tissues and cell types. Moreover, circulating miRNA expression shows a large variation between individuals. In this study, we did not investigate the underlying mechanism on how upregulation of miR-373-3p and downregulation of miR-194-5p in serum may cause early tumor progression during FOLFIRINOX.

A limitation of our study is that we did not include healthy controls and thus we are not able to determine whether serum miR-373-3p and miR-194-5p are differentially expressed compared to healthy individuals. Furthermore, we could not make a differentiation between the different stages of disease of the included patients due to the low numbers in the individual cohorts and response groups. Also, because of the relatively low number of patients in the discovery cohort (n=12), other miRNAs of importance might not have reached statistical significance and were therefore not selected for validation.

Further validation of the miRNAs described in this study in a larger patient cohort allows the distinction of subgroups of patients with PDAC, not only based on the stage of disease, but also based on tumor biology and treatment response. We have shown that miR-373-3p and miR-194-5p are significantly different between responding and non-

responding patients, already before the start of treatment and after one cycle of FOLFIRINOX. This suggests that response can be determined by using circulating biomarkers much earlier than with CT evaluation. Monitoring circulating miRNA expression could be a tool to select patients for available treatments, to spare patients from ineffective therapy, and to identify potential targets for future therapies.

CONCLUSION

High expression of miR-373-3p before the start of FOLFIRINOX and low expression of miR-194-5p after one cycle of FOLFIRINOX are associated with early tumor progression during treatment, but do not correlate with OS. This research shows new insights in the progression of PDAC, future clinical utility of miRNAs as predictive biomarkers, and possibly new therapeutic targets.

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SUPPLEMENTARY FILES

Supplementary Table 1. Differences in serum miRNA expression between stages of disease.

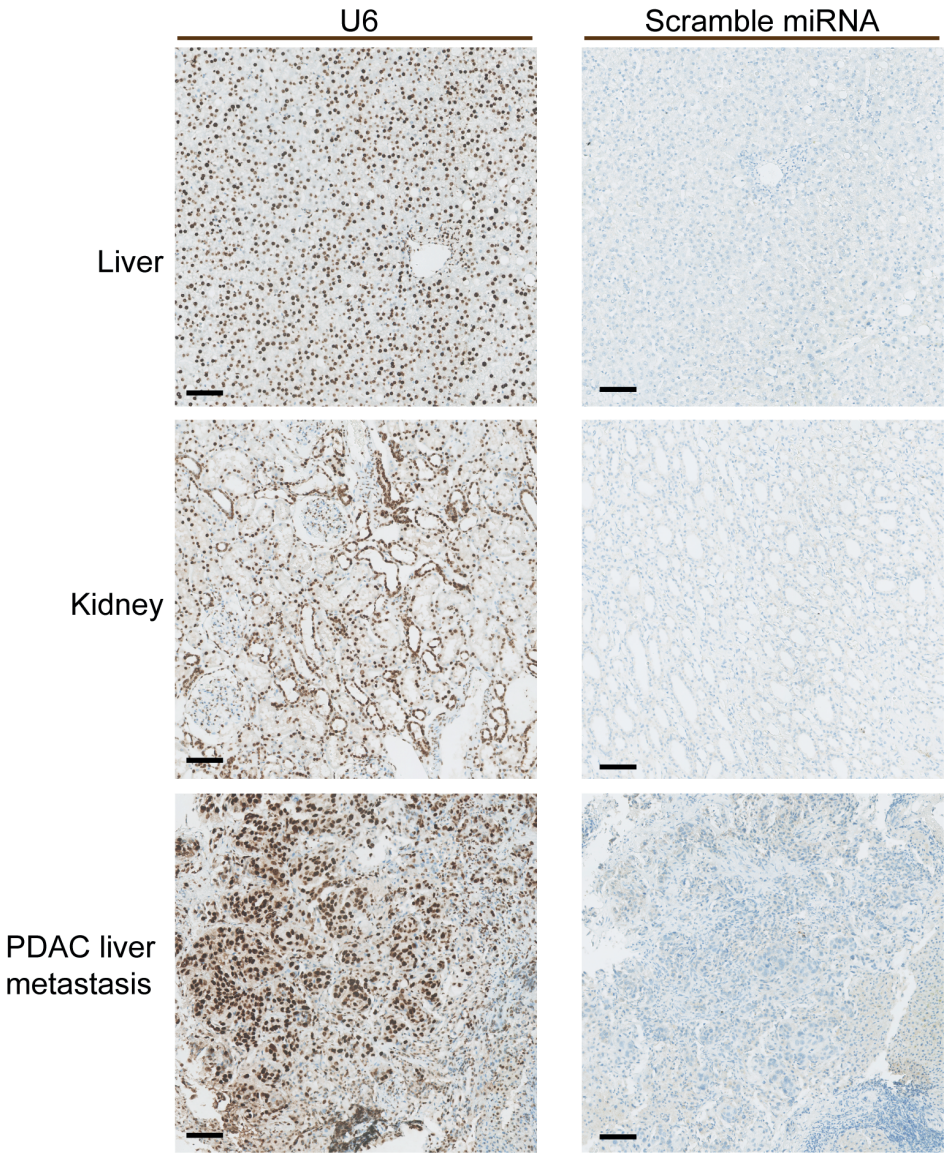
miRNA	Resectable disease (*n*=60) Log2 FD	LAPC (*n*=41) Log2 FD	Metastatic disease (*n*=19) Log2 FD	*P*
Before start of FOLFIRINOX				
hsa-miR-17-3p	Ref	-0.44	0.14	0.030
hsa-miR-373-3p	Ref	-0.29	-0.08	0.607
After one cycle of FOLFIRINOX				
hsa-miR-18a-5p	Ref	0.33	0.21	0.474
hsa-miR-194-5p	Ref	0.32	0.15	0.397
hsa-miR-24-3p	Ref	0.43	-0.03	0.198
hsa-miR-27a-3p	Ref	0.26	0.20	0.883

FD = fold difference, LAPC = locally advanced pancreatic cancer, hsa = homo sapiens (human), miR/miRNA = microRNA. *P*-values by one-way ANOVA.

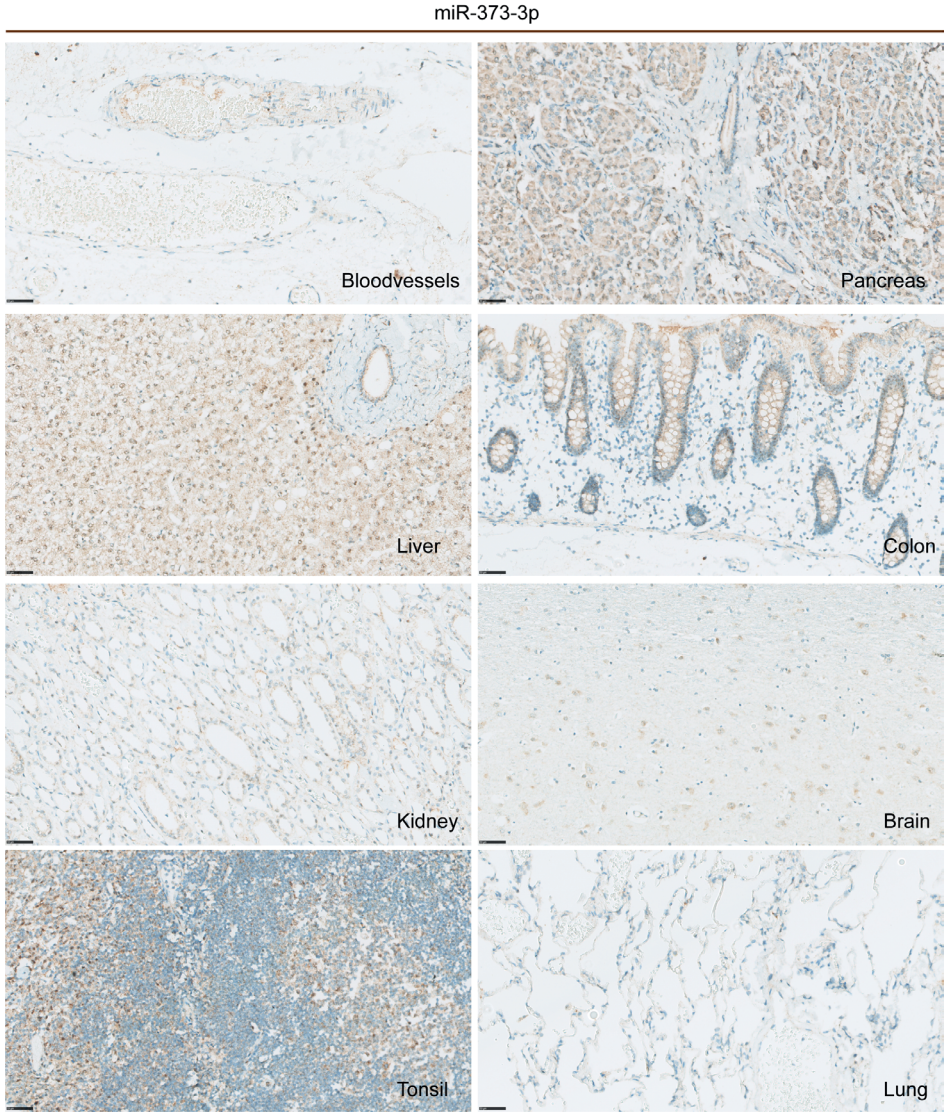
Supplementary Table 2. Differences in serum miRNA expression between patients with disease control and patients with progressive disease after FOLFIRINOX for the three different stages of disease.

miRNA	Resectable disease (*n*=60) Log2 FD*	*P*	LAPC (*n*=41) Log2 FD*	*P*	Metastatic disease (*n*=19) Log2 FD*	*P*
Before start of FOLFIRINOX						
hsa-miR-17-3p	0.58	0.040	0.09	0.829	-0.60	0.459
hsa-miR-373-3p	-2.16	0.566	1.56	<0.001	0.31	0.721
After one cycle of FOLFIRINOX						
hsa-miR-18a-5p	-0.03	0.874	0.03	0.880	0.04	0.900
hsa-miR-194-5p	-0.42	0.173	-0.36	0.434	-0.60	0.254
hsa-miR-24-3p	-0.13	0.689	-0.18	0.651	-0.40	0.401
hsa-miR-27a-3p	-0.54	0.287	-0.37	0.534	-0.79	0.354

FD = fold difference, LAPC = locally advanced pancreatic cancer, hsa = homo sapiens (human), miR/miRNA = microRNA. *Fold differences are presented as the miRNA expression of progressive disease patients compared to disease control patients as reference group. *P*-values by t-tests.



Supplementary Figure 1. In situ hybridization of liver, kidney, and pancreatic ductal adenocarcinoma (PDAC) liver metastasis tissue. Tissue sections are stained with U6 (positive control) or scramble miRNA probes (negative control), visualized with DAB. Scale bar = 100 µm.



Supplementary Figure 2. In situ hybridization of positive control tissues with miR-373-3p. Mir-373-3p expression is indicated in brown (DAB), cell nuclei are stained in blue with hematoxylin.

MiR-373-3p is expressed by normal endothelium, hepatocytes, colon epithelium, renal tubular cells, neurons (brain), and in tonsillar B cell germinal centers. MiR-373-3p is also expressed in pancreatic acinar cells, but not in normal pancreatic ductal cells. Scale bar = 50 µm.

Chapter 7

Serum miR-338-3p and miR-199b-5p are associated with the absolute neutrophil count in patients with resectable pancreatic cancer

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ABSTRACT

Background Several peripheral blood cell counts and immune ratios, such as the systemic immune-inflammation index (SII=platelet x neutrophil count/lymphocyte count) have prognostic value in patients with resectable pancreatic cancer (PDAC). Circulating microRNAs (miRNAs) are involved in various aspects of cancer and inflammation. We aimed to identify measurable circulating miRNAs in PDAC patients correlating with systemic inflammation parameters.

Methods A total of 42 PDAC patients was included in this study: twelve in the discovery (*n*=6 SII low; *n*=6 SII high) and 30 patients in the validation cohort (*n*=19 SII low, *n*=11 SII high). MiRNAs isolated from preoperative serum samples were measured with a 352 miRNA panel in the discovery cohort and individual miRNA primers in the validation cohort, using RT-qPCR (ID3EAL assays, MiRXES).

Results Only in the discovery cohort miR-328-3p, miR-338-3p, miR-1258, and miR-199b-5p were upregulated in high compared to low SII patients (fold difference ≥2, $P<0.05$). In the total cohort (*n*=42) correlations were found between miR-338-3p (r=0.48, $P=0.002$) and miR-199b-5p (r=0.44, $P=0.005$) and the absolute neutrophil count.

Conclusions Circulating miR-338-3p and miR-199b-5p are correlated to the neutrophil count in the blood of PDAC patients, suggesting a potential role of circulating miRNAs in cancer immune evasion and systemic inflammation.

INTRODUCTION

Pancreatic cancer (PDAC) has a poor prognosis. Only 10% of patients presents with stage I resectable disease and although their prognosis is much better compared to patients with advanced disease, 5-year overall survival (OS) of stage I disease is only 34%.[1] Moreover, the mortality and morbidity of pancreatic tumor resection is high[2] and 80% of resected patients have disease recurrence within two years.[3] Patient stratification for therapy, such as surgical resection, is important to prevent patients from receiving inefficacious treatment and related complications.

Several studies have shown that immune cell counts, for example absolute neutrophil or lymphocyte counts, and immune cell ratios in the peripheral blood are of prognostic and predictive value in PDAC[4, 5] The number of neutrophilic granulocytes is associated with a shorter OS in patients with resectable disease and locally advanced PDAC.[6] Similarly, the neutrophil-to-lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR), and the systemic immune-inflammation index (SII) are associated with poor prognosis in patients with resectable and non-resectable PDAC.[7-10] The SII was introduced by Hu *et al.* and is a ratio of the circulating platelet (P), neutrophil (N) and lymphocyte (L) counts (SII=P×N/L).[11] The reference value of the SII is 459, based on a population-based prospective cohort study of individuals in the Netherlands aged 65.9 years on average.[12] The SII was found to be a prognostic marker for cancer-specific survival and disease recurrence in resectable PDAC and seems to outperform other immune ratios, such as the NLR.[9, 10] A high SII (>900) before resection is associated with worse prognosis compared to a low SII (≤900) before resection.[9] However, the underlying mechanism of an altered immune system reflected by an increased SII leading to poorer prognosis in PDAC remains unclear.

Cell-free circulating microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression.[13] In the context of cancer, miRNAs play a role in several processes, such as apoptosis, cell proliferation, tumor suppression and immune response.[13-15] The majority of circulating miRNAs originates from endothelium and blood cells, including leukocytes and platelets.[16] Serum miRNAs mostly target immune related mRNAs.[17] Therefore, circulating miRNAs could reflect the status of the immune system.

In this study, we aim to identify differentially expressed miRNAs in the serum of resectable PDAC patients to explore the role of miRNAs in systemic inflammation.

MATERIALS AND METHODS

Patients and samples

Treatment-naïve patients with resectable PDAC were retrospectively identified based on availability of preoperative serum and absolute leukocyte and platelet counts. All patients underwent surgical resection of the primary tumor within the Erasmus University Medical Center Rotterdam in the period of February 2013-December 2017.

Peripheral venous blood samples from PDAC patients were collected preoperatively on the day of surgery or up to five days prior to surgery. This study was approved by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam for which all patients had provided written informed consent (MEC-2015-085). Blood was collected in serum tubes with clot activator of silica particles (BD, Franklin Lakes, NJ). None of the patients showed signs of infection that might indicate, for example, cholangitis or pancreatitis at time of blood collection. After collection, blood samples were centrifuged for ten minutes at 2000g, and serum was stored at -80°C until further use.

Absolute circulating neutrophil, lymphocyte, and platelet counts were measured as part of standard preoperative patient care. SII values were calculated as the absolute platelet count, multiplied by the absolute neutrophil to absolute lymphocyte ratio (SII=P×N/L). Patients were grouped as low SII (median 592.0, interquartile range 451.6-720.3) and high SII (median 1227.7, interquartile range (1054.4-2016.4)), based on the cutoff value of 900 determined by Aziz *et al.* SII values closest to the time of surgery were used for analysis. A total of 42 serum samples were available for this study. Twelve serum samples were used in the discovery cohort, six with the lowest SII and six with the highest SII, to select miRNAs for validation and correlation analyses. The remaining 30 serum samples, including nineteen with a low SII and nineteen with a high SII, were used in the validation cohort.

In this study, SII low patients were considered the control group. No other control samples were included, such as samples from chronic pancreatitis patients, because of the lack of evidence concerning the prognostic value of the SII in this patient group.

Serum miRNA isolation and quantitation

In the discovery cohort, RNA was isolated from 2×200 µL serum using the miRNeasy serum/plasma miRNA Isolation Kit (Qiagen, Hilden, Germany) in order to reach sufficient RNA volume for reliable measurement of 384 miRNA targets. In the validation cohort only 20 targets were measured and therefore RNA was isolated from 200 µL serum using the same isolation kit. In the validation cohort, two proprietary pre-mixed spike-in ~20

nucleotide control RNAs (MiRXES, Singapore) with sequences distinct from annotated mature human miRNAs (miRbase version 21) were added to the lysis buffer prior to the serum miRNA isolation according to manufacturer's instructions, in order to evaluate RNA isolation efficiency.

In the discovery cohort, serum miRNAs were reverse transcribed (RT) using ID3EAL miRNA-specific oligo's and RT spike-in RNA (MiRXES, Singapore) in a multiplex reaction per manufacturer's instruction. Complementary DNA (cDNA) was stored at -20 °C up to two weeks and thawed only once. cDNA was added to the ID3EAL miRNA qPCR Master Mix, containing buffer, polymerase and the passive reference dye ROX, and transferred to pre-loaded ID3EAL 384 Target Assay Panel plates (MiRXES, Singapore), including 352 individual quantitative polymerase chain reaction (qPCR) primers, 16 RT spike-ins and 16 inter-plate controls. PCR amplification was performed with the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Raw threshold cycle (Ct) values were determined using the 7500 Software (version 2.3; Applied Biosystems, Foster City, CA, USA).

In the validation cohort, selected serum miRNAs were measured with ID3EAL RT-qPCR after reverse transcription, using individual miRNA primers (MiRXES, Singapore). Raw Ct values were determined using the 7500 Software (version 2.3; Applied Biosystems, Foster City, CA, USA) and the serum miRNA samples were measured in duplicate.

Statistical analyses

Patient characteristics were compared using a Chi-square test for categorical and Mann-Whitney U test for numeric data. Progression-free survival (PFS) and cancer-specific survival (CSS) were calculated as the time between resection and radiologically confirmed progression or death caused by progression of the disease, respectively, comparing Kaplan-Meier curves with log-rank tests. Statistical analyses were performed with SPSS Statistics for Windows (version 24.0; IBM, Armonk, NY, USA) and Prism for Windows (version 5.01; GraphPad Software, San Diego, CA, USA).

In the discovery cohort, raw Ct values were normalized using reference miRNAs, spike-ins, and inter-plate calibrators, using an algorithm incorporated in the Cancer Panel Analysis Template (version 1.9, MiRXES, Singapore), provided by the manufacturer. The two miRNAs with the most stable expression among all samples were selected as reference miRNAs, using NormFinder software for Excel (version 0.953; MOMA, Aarhus University Hospital, Denmark). The cutoff values for detection of raw Ct values were set at a minimum of 9 and maximum of 33 cycles, based on the manufacturer's recommendation. The average miRNA expression of the low and high SII samples were compared

with a two-tailed t-test. Differentially expressed miRNAs between the high SII and low SII patients with a fold difference of ≤0.5 or ≥2, for downregulated or upregulated miRNAs respectively, and $P<0.05$, that were detectable within the Ct value limits in all 12 patient samples, were selected for validation and continuous correlation analyses.

In the validation cohort, raw Ct values were normalized using the same two reference miRNAs from the discovery cohort. miRNA expression relative to the reference miRNAs was calculated with the delta Ct method; expression=$2^{-(\text{Ct miRNA of interest-average Ct reference miRNAs})}$. The average miRNA expression was compared between patient groups using a two-tailed t-test.

Correlations between miRNA expression and SII or individual absolute blood cell counts were measured by Pearson's correlation coefficient, using all patient samples, measured with individual qPCR. An univariate Cox regression analysis was conducted to evaluate the association between miRNA expression and CSS and PFS.

RESULTS

Patient characteristics

A total of 42 treatment-naïve resectable PDAC patients were included in this study of whom serum was available and peripheral blood cell counts were measured before tumor resection at the Erasmus University Medical Center. No differences in patient characteristics were observed between the discovery cohort and validation cohort. In addition, no differences in patient characteristics were observed between SII low and SII high patients, except for the SII and absolute lymphocyte count, which was of course used to differentiate between patient groups. Patient characteristics are summarized in Table 1.

Differential serum miRNA expression in the discovery cohort

From the total cohort of PDAC patients, six patients with the highest SII values, and six patients with the lowest SII values were selected for serum miRNA discovery. Figure 1 shows a scheme of the miRNA study design. A list of miRNAs included in the discovery ID3EAL Cancer miRNA Knowledge panel can be found in Supplementary Table 1. Out of 352 miRNAs, 207 were detectable within the Ct limits in all twelve serum samples. Four differentially expressed miRNAs (DE miRNAs) showed a fold difference ≥2 and $P<0.05$ between the high SII and low SII group (Table 2). These four miRNAs were upregulated in the high SII group and were selected for validation in $n=30$ additional patients. In the miRNA discovery samples we found that miR-345-5p and miR-130b-5p had the best stability and were selected as reference miRNAs for the validation cohort.

Table 1. Patient characteristics.

	Discovery cohort (*n*=12)	**Validation cohort (*n*=30)**	***P***
Age (years), median (range)	70.0 (58-84)	69.5 (46-88)	0.466
Gender (%)			
Male	10 (83.3)	22 (73.3)	0.492
Female	2 (16.7)	8 (26.7)	
SII (%)			
Low	6 (50.0)	19 (63.3)	0.426
High	6 (50.0)	11 (36.7)	
SII, median (IQR)	1139.8 (398.5-2171.1)	744.0 (592.5-1051.3)	1.000
Absolute neutrophil count, median (IQR)	4.9 (3.4-8.1)	5.1 (4.4-5.6)	0.808
Absolute lymphocyte count, median (IQR)	1.3 (0.9-2.2)	1.7 (1.3-2.3)	0.353
Platelet count, median (IQR)	252.0 (183.8-346.8)	291.0 (238.0-326.0)	0.353
Bilirubin, median (IQR)	15.0 (7.5-54.3)	21.0 (9.5-29.5)	0.794
Tumor marker CA19-9, median (IQR)	200.0 (25.6-348.0)	47.0 (20.5-299.0)	0.258
Tumor location (%)			
Head	10 (83.3)	29 (96.7)	0.135
Body	0 (0)	1 (3.3)	
Tail	1 (8.3)	0 (0)	
Multifocal	1 (8.3)	0 (0)	
T-stage[a] (%)			
1	1 (8.3)	2 (6.7)	0.850
2	0 (0)	0 (0)	
3	11 (91.7)	28 (93.3)	
4	0 (0)	0 (0)	
N-stage[a] (%)			
0	4 (33.3)	7 (23.3)	0.505
1	8 (66.7)	23 (76.7)	
2	0 (0)	0 (0)	
Tumor differentiation (%)			
Good	0 (0)	0 (0)	0.395
Moderate	9 (75.0)	17 (56.7)	
Poor	3 (25.0)	10 (33.3)	
Unknown	0 (0)	3 (10.0)	
Adjuvant chemotherapy[b] (%)	6 (50.0)	10 (33.3)	0.315
CSS (months), median (95% CI)	24.9 (9.5-40.3)	18.2 (15.2-21.2)	0.477
PFS (months), median (95% CI)	19.6 (6.6-32.6)	13.2 (11.7-14.8)	0.233

SII = systemic immune-inflammation index, IQR = interquartile range, CSS = cancer-specific survival, CI = confidence interval, PFS = progression free survival. [a] T- and N-stage classification according to AJCC 7th edition. [b] Standard adjuvant chemotherapy consisted of six cycles of gemcitabine.

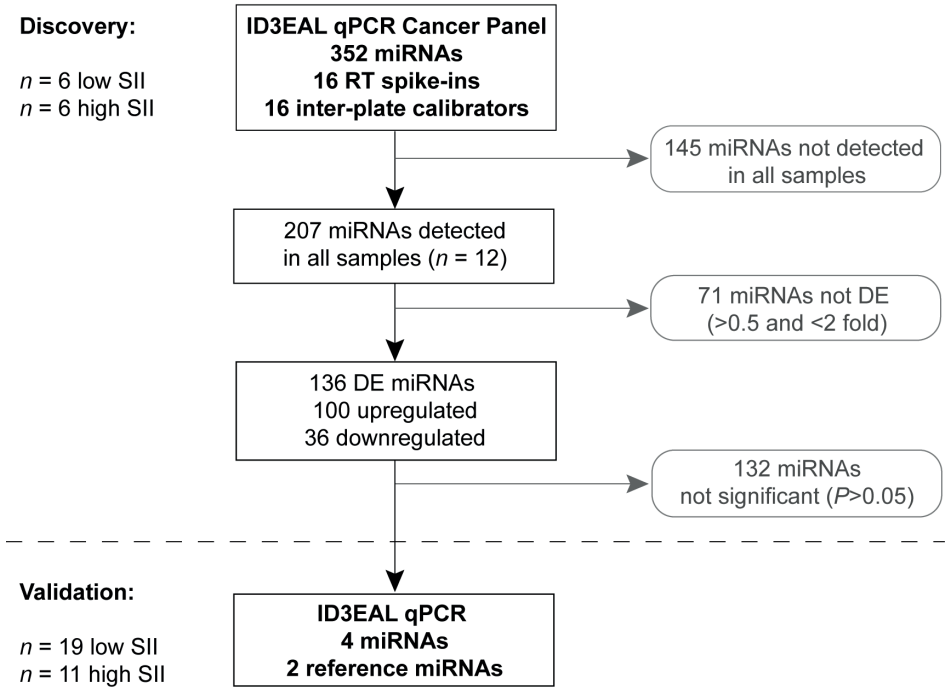

Figure 1. Schematic overview of serum miRNA analysis. In the discovery cohort *n*=12 patients with resectable pancreatic cancer with low or high systemic immune-inflammation index (SII) were analyzed using an RT-qPCR based 352 cancer miRNA panel from MiRXES to identify differentially expressed miRNAs (DE miRNAs). Statistical significance was calculated using a two-tailed t-test. Four DE miRNAs were measured in the validation cohort. RT = reverse transcription.

Table 2. Differentially expressed serum miRNAs in high SII (*n*=6) versus low SII (*n*=6) patients in the discovery cohort that were selected for validation.

microRNA	Fold difference	*P*
hsa-miR-328-3p	2.46	0.006
hsa-miR-338-3p	2.62	0.044
hsa-miR-1258	7.83	0.028
hsa-miR-199b-5p	2.01	0.022

Serum miRNA expression in the validation cohort

In one out of 30 patient samples of the validation cohort we could not detect the four miRNAs of interest or the reference miRNAs by RT-qPCR and this sample was therefore excluded from further analysis. MiR-1258 was not detected in 10/60 measurements (17%), in both the low SII as well as the high SII patients. None of the four selected

miRNAs remained significantly upregulated between high SII and low SII patients in the independent validation set (Table 3). In contrast to our findings in the discovery cohort, miR-328-3p showed a significant downregulation in patients with high SII compared to low SII (*P*=0.022).

Table 3. Differences in serum expression of the four selected miRNAs in high SII (*n*=11) versus low SII (*n*=19) patients of the validation cohort.

microRNA	Fold difference	*P*
hsa-miR-328-3p	0.52	0.022
hsa-miR-338-3p	1.20	0.252
hsa-miR-1258	0.54	0.349
hsa-miR-199b-5p	1.12	0.662

Serum miRNA expression association with blood cell counts and survival

In addition to using an SII cutoff of 900 to separate the two patient groups, we calculated the correlations between expression data of miR-338-3p and miR-199b-5p and the SII and individual immune cell counts (Figure 2). Two miRNAs were excluded from these analyses: miR-328-3p was excluded due to discrepancy in expression directionality between the discovery and validation cohort and miR-1258 was excluded due to the lack of detection.

There was a significant correlation between serum miR-338-3p (Figure 2a) and the absolute neutrophil count (r=0.48, *P*=0.002) as well as between serum miR-199b-5p (Figure 2b) and the absolute neutrophil count (r=0.44, *P*=0.005). Neither miR-338-3p or miR-199b-5p showed a significant correlation between serum expression and the SII (*P*=0.185 and *P*=0.145), the absolute lymphocyte count (*P*=0.453 and *P*=0.280) or absolute platelet count (*P*=0.576 and *P*=0.918).

To evaluate whether miR-338-3p and miR-199b-5p also show a correlation with survival, univariate Cox regression analyses were performed. There were no significant correlations between miR-338-3p and CSS (HR=2.672, *P*=0.549) or PFS (HR=4.384, *P*=0.350) or between miR-199b-5p and CSS (HR=2.241, *P*=0.705) or PFS (HR=5.279, *P*=0.432), as shown in Table 4.

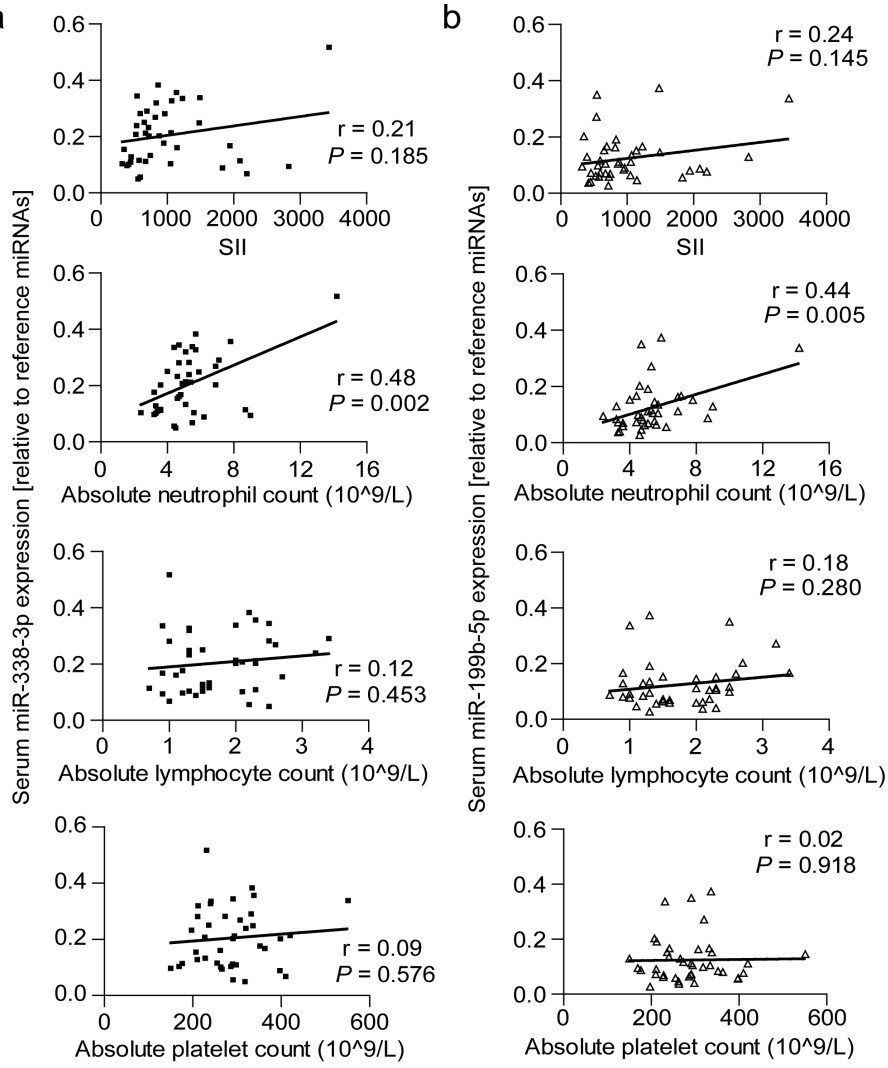

Figure 2. Continuous relationship between serum miR-338-3p (a) and miR-199b-5p (b) expression and the systemic immune-inflammation index (SII) or individual absolute blood cell counts in the total cohort of patients (*n*=42). Serum miRNA expression is relative to the average expression of reference miRNAs. r=correlation coefficient, *P*-value is calculated by Pearson's correlation.

Table 4. Univariate Cox regression analysis of miR-338-3p and miR-199b-5p expression for cancer-specific survival (CSS) and progression-free survival (PFS) in patients with resectable pancreatic cancer.

microRNA	Hazard ratio* for CSS (95% CI)	*P*	Hazard ratio* for PFS (95% CI)	*P*
hsa-miR-338-3p	2.672 (0.107-66.579)	0.549	4.384 (0.198-97.144)	0.350
hsa-miR-199b-5p	2.241 (0.035-145.355)	0.705	5.279 (0.083-333.937)	0.432

* Hazard ratio for every 1 unit change, where each unit represents a change of 1.0 fold difference over reference miRNA expression. CI = confidence interval.

DISCUSSION

PDAC progression has been linked with the host's systemic inflammatory response and circulating blood cells.[6, 9] This is the first study that attempts to identify a circulating miRNA signature related to the altered SII and immune cell counts in PDAC patients. MiRNAs are heavily involved in immune cell proliferation, differentiation and function.[17-19] Cell-free miRNAs in the circulation are primarily derived from blood cells and the endothelium.[16] Circulating miRNAs have potential as cancer biomarkers due to their stability in plasma or serum, their roles in cell-cell contact, and regulation of cellular processes.[20] Although no correlation between the SII and any of the four miRNAs could be validated in our cohort, there was a statistically significant correlation between miR-338-3p and miR-199b-5p expression and the absolute neutrophil count. Whether miR-338-3p and miR-199b-5p are also upregulated in circulating neutrophils or tumor infiltrating neutrophils in PDAC patients needs to be investigated in the near future.

Neutrophilia is associated with a poor prognosis in PDAC,[6, 21] but the underlying mechanism is unknown. The chronic state of inflammation may well lead to an influx of immunosuppressive neutrophils into tumors[22] that recruit regulatory T cells in the tumors.[23] Immunosuppressive neutrophils generate high levels of reactive oxygen species (ROS) which blocks T cell proliferation[24] and can also induce CD8 T cell apoptosis through secretion of nitric oxide.[25] MiR-338-3p has not yet been described for its association with neutrophils or PDAC. However, miR-338-3p expression was found to be upregulated in leukocytes derived from patients with sporadic amyotrophic lateral sclerosis, showing that the disease related transcriptional miRNA alterations exist in tissues outside of the affected organ system.[26] Upregulation of miR-338-3p was also found in peripheral blood mononuclear cells of patients with pemphigus vulgaris, where it suppresses cell viability.[27] Whereas miR-338-3p is upregulated in the circulation in a limited number of studies, miR-338-3p is often downregulated in cancer tissues. Because of its tumor suppressing function, downregulation of miR-338-3p in cancer tissue samples was proven to be a poor prognostic factor in several cancers, such as gastric, and prostate cancer.[28, 29]

In a recent study miR-199b-5p expression in PDAC tissue was also linked with poor prognosis.[30] However, it remains unclear whether miR-199b-5p originates from the cancer cells themselves or from other cell types. Others have shown that the miR-199 family, including miR-199b, is primarily expressed in the fibrotic tumor associated stroma in PDAC tissues.[31] miR-199b findings are inconsistent across studies in different cancers. For instance in cervical cancer, miR-199b-5p overexpression is associated with poor prognosis and metastasis,[32] while in other cancers such as breast, and colorectal cancer miR-199b-5p has a tumor suppressor function and poor prognosis is reflected by a

decrease of expression.[33, 34] MiR-199b is overexpressed in myeloid progenitor cells and expression levels drop along with differentiation of these cells.[35] While in some studies miR-199b levels are reportedly upregulated in plasma of acute myeloid leukemia (AML) patients compared to healthy controls,[36] in other studies high levels of miR-199b were found favorable in terms of OS of AML patients.[35] In sepsis patients, miR-199b-5p was specifically upregulated in blood cells (including neutrophils), but this difference in expression could not be detected in the serum.[37] Until now, the exact origin of disease-related circulating miRNAs remains unclear. Once in the circulation, miRNAs are transferred to various cell types where they can alter gene expression depending on the cellular context and targeted organ.[38, 39]

MiRNA target prediction using the online database miRDB[40] showed that in general miR-338-3p has 601 predicted mRNA targets. The gene with the highest target score for miR-338-3p is Casitas B-lineage Lymphoma Proto-oncogene (*CBL*), which is a key player in balancing of the immune response and considered a potential target for anticancer immune therapy.[41] In neutrophils specifically, c-CBL mediates the termination of FcγRIIa signaling, resulting in downregulation of neutrophil function.[42] For miR-199b-5p there are 556 predicted mRNA targets, from which Discoidin Domain Receptor Tyrosine Kinase 1 (*DDR1*) has the highest target score. *DDR1* is known as a collagen receptor on leukocytes which plays a role in the physical attachment of leukocytes to collagen, as well as the differentiation and cytokine/chemokine production of cells of the myeloid lineage.[43] These findings suggest that miR-338-3p and miR-199b-5p could exert pivotal roles in the regulation of immune related gene expression.

The ID3EAL qPCR assay from MiRXES used in our study is a sensitive method for measuring serum miRNAs. This method utilizes miRNA-specific reverse transcription as well as a combination of a miRNA-specific forward and reverse primers. Two hundred and seven miRNAs included in the 352 miRNA panel were detected in all twelve discovery serum samples. This is higher than in qPCR microarrays without the use of miRNA-specific RT primers and is comparable to more advanced techniques, such as next generation sequencing and digital count technologies.[44]

The main limitation of this retrospective study is the low number of patient samples. All patient samples had previously been collected from treatment-naïve patients, as part of our local blood sample biobank. Despite the large number of collected blood samples, only 42 serum samples were available for this study, due to missing data on absolute immune cells counts. For future studies, we are aiming to prospectively collect all data on, for example, immune cell counts, immune cell ratios and tumor markers in addition to blood samples for circulating biomarker analyses.

A significant prognostic value of the SII or circulating neutrophil counts could not be confirmed in this experiment. In addition, none of the investigated miRNAs showed an association between expression levels and survival, although there is a trend visible towards increased hazard ratios for CSS and PFS with increased expression of miR-338-3p and miR-199b-5p. The small sample size, causing wide confidence intervals, could explain the lack of statistical significance. Another unexpected finding is the discrepancy of miR-328-3p expression between the discovery and validation cohort, which might be explained by the high variability of miRNA expression in low SII patients. Although the SII cutoff value of 900 has been validated for its prognostic value in multiple studies, the SII might not be a good marker to differentiate between patient subgroups on a molecular level. However, the four miRNAs in the validation cohort of this study were selected based on the differential expression between low and high SII dichotomization. In retrospect, miRNA selection could have been improved by using, for example, the absolute neutrophil count to distinguish the two patient groups.

In conclusion, we aimed to identify serum miRNA signatures that are related to systemic inflammation in PDAC patients. Serum miR-338-3p and miR-199b-5p were found to be significantly correlated to the absolute neutrophil count in patients with resectable PDAC. Our findings support the potential role of miRNAs in the altered immune system of pancreatic cancer patients. Future research regarding circulating miRNAs in PDAC patients could elucidate whether miRNAs can serve as therapeutic target by altering immune related intercellular communication and reversing cancer immune evasion.

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SUPPLEMENTARY FILES

Supplementary Table 1. List of miRNAs included in the ID3EAL Cancer miRNA Knowledge panel (MiRXES, Singapore).

hsa-let-7c-5p	hsa-miR-497-5p	hsa-miR-98-5p	hsa-miR-574-3p
hsa-miR-150-5p	hsa-miR-544a	hsa-miR-203a-3p	hsa-miR-214-5p
hsa-let-7g-5p	hsa-miR-671-5p	hsa-miR-140-5p	hsa-miR-490-5p
hsa-miR-153-3p	hsa-miR-361-3p	hsa-miR-302a-3p	hsa-miR-1271-5p
hsa-miR-193a-3p	hsa-miR-1246	hsa-miR-378a-5p	hsa-miR-26a-5p
hsa-miR-323a-3p	hsa-miR-30a-5p	hsa-miR-429	hsa-miR-30d-5p
hsa-miR-483-3p	hsa-miR-215-5p	hsa-miR-506-3p	hsa-let-7i-5p
hsa-miR-516b-5p	hsa-miR-128-3p	hsa-miR-421	hsa-miR-190a-5p
hsa-miR-564	hsa-miR-136-5p	hsa-miR-129-2-3p	hsa-miR-369-3p
hsa-miR-146a-3p	hsa-miR-383-5p	hsa-miR-708-5p	hsa-miR-423-3p
hsa-miR-885-3p	hsa-miR-433-3p	hsa-miR-17-3p	hsa-miR-526a
hsa-let-7d-5p	hsa-miR-515-5p	hsa-miR-29b-3p	hsa-miR-602
hsa-miR-196a-5p	hsa-miR-92b-3p	hsa-miR-211-5p	hsa-miR-221-5p
hsa-miR-15b-5p	hsa-miR-765	hsa-miR-142-3p	hsa-miR-500a-5p
hsa-miR-191-5p	hsa-miR-193b-5p	hsa-miR-301a-3p	hsa-miR-1258
hsa-miR-194-5p	hsa-miR-1260a	hsa-miR-330-3p	hsa-miR-27a-3p
hsa-miR-326	hsa-miR-31-5p	hsa-miR-452-5p	hsa-miR-139-5p
hsa-miR-146b-5p	hsa-miR-223-3p	hsa-miR-21-5p	hsa-miR-1-3p
hsa-miR-519a-3p	hsa-miR-135a-5p	hsa-miR-10b-3p	hsa-miR-195-5p
hsa-miR-608	hsa-miR-154-5p	hsa-miR-155-3p	hsa-miR-372-3p
hsa-miR-29c-5p	hsa-miR-340-3p	hsa-miR-147b	hsa-miR-425-3p
hsa-miR-301b-3p	hsa-miR-323b-5p	hsa-miR-19b-3p	hsa-miR-520c-3p
hsa-miR-16-5p	hsa-miR-515-3p	hsa-miR-106a-5p	hsa-miR-636
hsa-miR-199a-5p	hsa-miR-558	hsa-miR-218-5p	hsa-miR-223-5p
hsa-miR-27b-3p	hsa-miR-181a-2-3p	hsa-miR-9-3p	hsa-miR-625-3p
hsa-miR-23b-3p	hsa-miR-532-3p	hsa-miR-99b-5p	hsa-miR-664a-3p
hsa-miR-302d-3p	hsa-miR-320d	hsa-miR-337-3p	hsa-let-7f-5p
hsa-miR-345-5p	hsa-miR-99a-5p	hsa-miR-485-5p	hsa-miR-7-5p
hsa-miR-494-3p	hsa-miR-200b-3p	hsa-miR-552-3p	hsa-miR-331-3p
hsa-miR-499a-5p	hsa-miR-152-3p	hsa-miR-181c-3p	hsa-miR-127-3p
hsa-miR-657	hsa-miR-184	hsa-miR-219a-2-3p	hsa-miR-200a-3p
hsa-miR-34c-3p	hsa-miR-328-3p	hsa-miR-374b-5p	hsa-miR-377-3p
hsa-miR-1228-5p	hsa-miR-409-3p	hsa-miR-20a-5p	hsa-miR-451a
hsa-miR-24-3p	hsa-miR-518b	hsa-miR-192-5p	hsa-miR-525-5p
hsa-miR-199a-3p	hsa-miR-561-3p	hsa-miR-181a-5p	hsa-miR-615-3p
hsa-miR-30b-5p	hsa-miR-144-5p	hsa-miR-125a-5p	hsa-miR-140-3p
hsa-miR-126-5p	hsa-miR-885-5p	hsa-miR-361-5p	hsa-miR-616-3p

Supplementary Table 1. List of miRNAs included in the ID3EAL Cancer miRNA Knowledge panel (MiRXES, Singapore). (continued)

hsa-miR-376a-3p	hsa-miR-1915-3p	hsa-miR-324-3p	hsa-miR-18a-5p
hsa-miR-18b-5p	hsa-let-7e-5p	hsa-miR-491-5p	hsa-miR-10b-5p
hsa-miR-495-3p	hsa-miR-28-5p	hsa-miR-568	hsa-miR-122-5p
hsa-miR-510-5p	hsa-miR-10a-5p	hsa-miR-196a-3p	hsa-miR-146a-5p
hsa-miR-542-5p	hsa-miR-130a-3p	hsa-miR-340-5p	hsa-miR-34c-5p
hsa-miR-296-3p	hsa-miR-200c-3p	hsa-miR-939-5p	hsa-miR-339-5p
hsa-miR-1206	hsa-miR-373-3p	hsa-miR-9-5p	hsa-miR-410-3p
hsa-miR-26b-5p	hsa-miR-448	hsa-miR-129-5p	hsa-miR-517a-3p
hsa-miR-208a-3p	hsa-miR-504-5p	hsa-miR-224-5p	hsa-miR-625-5p
hsa-miR-125b-5p	hsa-miR-449b-5p	hsa-miR-17-5p	hsa-miR-143-5p
hsa-miR-126-3p	hsa-miR-125a-3p	hsa-miR-367-3p	hsa-miR-628-5p
hsa-miR-382-5p	hsa-miR-298	hsa-miR-338-3p	hsa-miR-19a-3p
hsa-miR-449a	hsa-miR-15a-5p	hsa-miR-498	hsa-miR-34a-5p
hsa-miR-124-3p	hsa-miR-105-5p	hsa-miR-99b-3p	hsa-miR-202-3p
hsa-miR-149-5p	hsa-miR-217	hsa-miR-216b-5p	hsa-miR-590-5p
hsa-miR-130b-3p	hsa-miR-145-5p	hsa-miR-22-3p	hsa-miR-22-5p
hsa-miR-335-5p	hsa-miR-29c-3p	hsa-miR-181c-5p	hsa-miR-337-5p
hsa-miR-484	hsa-miR-371a-3p	hsa-miR-222-3p	hsa-miR-1202
hsa-miR-520h	hsa-miR-200a-5p	hsa-miR-106b-5p	hsa-miR-96-5p
hsa-miR-637	hsa-miR-193b-3p	hsa-miR-378a-3p	hsa-miR-212-3p
hsa-miR-193a-5p	hsa-miR-601	hsa-miR-324-5p	hsa-miR-143-3p
hsa-miR-629-5p	hsa-let-7i-3p	hsa-miR-519d-3p	hsa-miR-155-5p
hsa-miR-25-3p	hsa-miR-423-5p	hsa-miR-595	hsa-miR-376c-3p
hsa-miR-103a-3p	hsa-miR-1304-5p	hsa-miR-425-5p	hsa-miR-20b-5p
hsa-miR-132-3p	hsa-let-7a-5p	hsa-miR-362-3p	hsa-miR-492
hsa-miR-185-5p	hsa-miR-93-5p	hsa-miR-208b-3p	hsa-miR-592
hsa-miR-30e-5p	hsa-miR-205-5p	hsa-miR-29a-3p	hsa-miR-129-1-3p
hsa-miR-133b	hsa-miR-144-3p	hsa-miR-183-5p	hsa-miR-151a-5p
hsa-miR-486-5p	hsa-miR-302b-3p	hsa-miR-138-5p	hsa-miR-1290
hsa-miR-493-3p	hsa-miR-151a-3p	hsa-miR-296-5p	hsa-miR-374a-5p
hsa-miR-769-5p	hsa-miR-489-3p	hsa-miR-379-5p	hsa-miR-148b-3p
hsa-miR-34b-3p	hsa-miR-539-5p	hsa-miR-450a-5p	hsa-miR-519c-3p
hsa-miR-874-3p	hsa-miR-630	hsa-miR-517c-3p	hsa-miR-570-3p
hsa-miR-92a-3p	hsa-miR-7-1-3p	hsa-miR-605-5p	hsa-miR-663a
hsa-miR-182-5p	hsa-miR-455-3p	hsa-miR-21-3p	hsa-miR-106b-3p
hsa-miR-133a-3p	hsa-let-7b-5p	hsa-miR-342-5p	hsa-miR-190b
hsa-miR-186-5p	hsa-miR-100-5p	hsa-miR-1226-3p	hsa-miR-221-3p
hsa-miR-30e-3p	hsa-miR-210-3p	hsa-miR-30a-3p	hsa-miR-147a
hsa-miR-196b-5p	hsa-miR-134-5p	hsa-miR-187-3p	hsa-miR-219a-5p

Supplementary Table 1. List of miRNAs included in the ID3EAL Cancer miRNA Knowledge panel (MiRXES, Singapore). (continued)

hsa-miR-511-5p	hsa-miR-370-3p	hsa-miR-141-3p	hsa-miR-320a
hsa-miR-582-5p	hsa-miR-135b-5p	hsa-miR-363-3p	hsa-miR-375
hsa-miR-802	hsa-miR-432-5p	hsa-miR-381-3p	hsa-miR-509-3p
hsa-miR-130b-5p	hsa-miR-487b-3p	hsa-miR-329-3p	hsa-miR-526b-5p
hsa-miR-1228-3p	hsa-miR-638	hsa-miR-503-5p	hsa-miR-584-5p
hsa-miR-95-3p	hsa-miR-34a-3p	hsa-miR-612	hsa-miR-363-5p
hsa-miR-199b-5p	hsa-miR-589-5p	hsa-miR-31-3p	hsa-miR-142-5p
hsa-miR-137	hsa-miR-23a-3p	hsa-miR-486-3p	hsa-miR-365a-3p
hsa-miR-188-5p	hsa-miR-30c-5p	hsa-miR-320c	hsa-miR-342-3p
hsa-miR-302c-3p	hsa-miR-181a-3p	hsa-miR-32-5p	hsa-miR-376b-3p
hsa-miR-424-5p	hsa-miR-206	hsa-miR-204-5p	hsa-miR-455-5p
hsa-miR-613	hsa-miR-139-3p	hsa-miR-491-3p	hsa-miR-449c-5p

Part III

Immunologic biomarkers for prediction of treatment response and prognosis

Chapter 8

Circulating immunological biomarkers: prognosis of pancreatic cancer patients reflected by the immune system

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ABSTRACT

To date, little advances have been made towards new and more effective therapies for pancreatic ductal adenocarcinoma (PDAC). Discovery of prognostic and predictive biomarkers is needed to stratify patients for available treatments and to elucidate how new therapies could be developed. Recent studies have made clear that the immune system is not only affected in the microenvironment of the primary tumor, it is also systemically disrupted in PDAC patients. Under normal circumstances the immune system is in perfect balance with both pro- and anti-inflammatory components present. In this review we focus on circulating immunological characteristics including immune cells and their subtypes, cytokines, and immune checkpoints in the peripheral blood, not only to understand the poor prognosis of PDAC patients, but also to find new leads for new innovative therapies.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with poor prognosis.[1] Patients are diagnosed at a late stage of disease,[2] and the biological behavior of PDAC results in rapid tumor progression. In contrast to the majority of other solid cancer types, few advances have been made towards new therapeutic options for PDAC. Current treatment strategies include surgery, radiotherapy, and systemic chemotherapy, but have limited success. Therefore, the five-year survival rate is less than 10%.[2] There is a great need for biomarkers that can stratify PDAC patients for available treatments and predict treatment response and survival. At the same time, these biomarkers could form a basis for the development of new therapies.

Currently, 'liquid biopsies' are gaining more interest in PDAC biomarker research for several reasons. First, representative tumor biopsies to investigate the molecular characteristics of the cancer cells or the immune component of the disease are often difficult to obtain. Second, the intratumoral heterogeneity of PDAC is not reflected by a single biopsy, which is a crucial limitation of PDAC tissue analyses. Blood samples, including whole blood, and isolated plasma and serum, may represent a more complete molecular make-up of a primary tumor and its distant metastases.[3] Peripheral blood can be sampled repeatedly with little safety risks, providing a valuable source to investigate biomarkers over the course of the disease. Circulating biomarkers show important limitations, though. Circulating tumor mutations, for example, are not detectable in all PDAC blood samples, with detection rates up to 69%,[4] detection rates of circulating tumor cells ranges from 11-92%.[5] Also, whether or not circulating immune characteristics represent immunological changes within the tumor microenvironment is not absolutely clear yet.

Cancer has been linked to chronic inflammation since the nineteenth century. Chronic inflammation can induce malignancy and cancer, in turn, promote inflammatory responses at the same time. Although tumor-induced inflammation could be a defense mechanism with tumor-suppressing purposes, the effect is often limited or even opposite, especially in PDAC.[6] The rich stroma surrounding PDAC tumors, including active cancer-associated fibroblasts (CAFs), partially prevents anti-tumor immune cells from infiltrating the tumor and forms a tumor-promoting environment that supports cancer proliferation. Therefore, upregulation of the immune system shows little effect on the well protected cancer cells. Interestingly, despite the 'cold' nature of PDAC, it does in fact induce systemic immune responses. Impairment of the innate and adaptive immune responses, reflected by systemic inflammation markers, can be detected even prior to PDAC diagnosis.[7]

We have performed a review of current literature on immunological characteristics in the circulation of PDAC patients that may serve as biomarkers to monitor therapy response and predict prognosis, and that could help identify immunological targets for new therapies.

IMMUNE CELLS

Immune cell counts and immune cell ratios are the most extensively investigated types of immunological markers in PDAC. Absolute cell counts in whole blood are easy to measure and often available in diagnostic settings. Many types of immune cells have been linked to prognosis. Elevated levels of neutrophils,[8, 9] monocytes,[10, 11] and regulatory T cells (Tregs)[12, 13] are a poor prognostic factor for survival, while elevated levels of total lymphocytes,[14-16] dendritic cells (DCs),[17] and cytotoxic T cells[13] in the circulation are associated with favorable outcomes. However, immune cell functions are often ambiguous and cell counts are not always associated with solely good or poor outcome. Table 1 gives an overview of both tumor-suppressing as well as tumor-promoting functions of different immune cells.

The negative effect of elevated neutrophil counts on survival seems contradictory. An elevated count could indicate an activated immune system, fighting the cancer cells. Neutrophils are usually the first responders at sites of inflammation and infection and immediately ready to kill foreign particles. However, neutrophilia in PDAC is associated with poor overall survival (OS),[9] and with poor progression-free survival (PFS).[18] Chemotherapy-induced neutropenia in patients with advanced PDAC is correlated with better OS.[8] The inverse correlation between neutrophilia and survival in patients with PDAC might be explained by an altered balance of pro- and anti-inflammatory cytokines induced by tumors. Normally, neutrophils differentiate into an N1 anti-tumor phenotype under the influence of type I interferons. However, in an environment rich in transforming growth factor beta (TGF-β), neutrophils tend to differentiate into the N2 phenotype with tumor-promoting effects. An increase in N2 neutrophils will stimulate cancer cell growth, angiogenesis and metastasis through the secretion of vascular endothelial growth factor (VEGF).[19] Pancreatic cancer cells are known to produce TGF-β as well, which is released into the tumor's microenvironment.[20] The enhanced N2 polarization of neutrophils by TGF-β may explain the link between neutrophilia and poor prognosis of PDAC patients. To our knowledge, there is currently no literature on other types of circulating granulocytes, e.g. eosinophils of basophils, and their role in PDAC, but this could be an interesting topic of research in the future.

Table 1. Tumor-suppressive and tumor-promoting functions of immune cells involved in pancreatic cancer.

Immune cell	Tumor-suppressive functions	Tumor-promoting functions
B cells[22]	Secretion of antibodies Antigen presentation	Production of tumor growth factors Production of TGF-β, causing: N2 polarization of neutrophils Conversion of naïve T cells into Tregs Inhibition of Th1 and CD8+ cells
Cytotoxic T cells[25]	Pro-inflammatory cytokine production Cytotoxic granule release, causing apoptosis of cells Cell-cell binding induced apoptosis	
Dendritic cells[29, 31]	Mature DCs: Antigen presentation	Immature DCs: Angiogenesis Cancer cell growth
Gamma delta T cells[24]	Activation and maturation of DCs Priming cytotoxic T cells Stimulation of B cell antibody production Induction of NK cells	Mobilization of MDSCs Inhibition CD8+ cells Cancer cell growth Angiogenesis
Helper T cells[27]	Th1: Production of IFN-γ Priming cytotoxic T cells Induction of NK cells Th2: Accumulation of granulocytes Stimulation of B cell antibody production	Th17: IL-17 production Activation of Tregs Mobilization of MDSCs Cancer cell growth
Macrophages[20]	M1 phenotype, e.g. under influence of IFN-γ: Antigen presentation Phagocytosis Pro-inflammatory cytokine production Production of cytotoxic ROS and NO	M2 phenotype, e.g. under influence of IL-4: Anti-inflammatory cytokine and angiogenic protein production Formation of extracellular matrix
Myeloid-derived suppressor cells[33]		Suppression of CD8+ T cells, NK cells, DCs and macrophages Impaired lymphocyte homing Activation of inhibitory immune checkpoint molecules
Neutrophils[19]	N1 phenotype, under influence of type I interferons: Pro-inflammatory cytokine production Phagocytosis Degranulation	N2 phenotype, under influence of TGF-β: Anti-inflammatory cytokine production Production of VEGF, causing: Cancer cell growth Angiogenesis, Metastasis
Natural killer cells[28]	Release of cytotoxic granules, causing lysis of cells Pro-inflammatory cytokine secretion, e.g. IFN-γ, leading to activation of macrophages	
Regulatory T cells[23]		Inhibition of cytotoxic T cells, causing: Tolerance to auto-antigens Cancer cell growth

CD = cluster of differentiation, DC = dendritic cell, IFN = interferon, MDSC = myeloid-derived suppressor cell, NK = natural killer, NO = nitric oxide, ROS = reactive oxygen species, TGF = transforming growth factor, Th = T helper, VEGF = vascular endothelial growth factor.

Low circulating lymphocyte counts at diagnosis[15, 16, 21] and during treatment[14] reflect immune suppression – and therefore a diminished anti-tumor response. When this occurs, PDAC tumor cells can escape from host immune responses. The total lymphocyte count is inversely correlated with the stage of the disease.[16] It is not clear, however, if tumor progression induces a decrease in lymphocytes or if a low lymphocyte count provides a tumor-promoting environment, leading to tumor progression. Low circulating lymphocyte counts are associated with shorter survival.[14, 15]

The total lymphocyte compartment consists of various immune cell subpopulations of T cells, B cells, and natural killer cells (NK cells). Each subpopulation has its own role in cancer surveillance. Therefore, it is important to study how individual subpopulations of circulating lymphocytes correlate to prognosis. B cells, for example, seem to be tumor-promoting in patients with resectable PDAC and are negatively associated with OS.[13] B cells can produce tumor growth factors, such as interleukin (IL)-10, which facilitates and promotes tumor development.[22] In addition, regulatory B cells can inhibit effector T cells. B cells can also produce TGF-β, causing not only N2 polarization of neutrophils but also the conversion of naïve CD4+ T cells into CD4+ Foxp3+ Treg cells and inhibition of, among other cells, T helper 1 (Th1) cells and CD8+ T cells, thereby facilitating a more tumor-friendly environment.[22] Next to anti-tumor antibody production, B cells can produce autoantibodies. These autoantibodies can form circulating immune complexes, depositing in cancer tissue, that are associated with poor prognosis. Not only do these antibodies lack a normal anti-tumor immune response initiation, but they trigger the complement pathway, leading to an proangiogenic tumor environment, and activate myeloid-derived suppressor cells,[22] which will be discussed at the end of this section.

Regulatory T cells (CD4+) or suppressor T cells are naturally immunosuppressive as they maintain tolerance to autoantigens. Unfortunately, Tregs also inhibit effector T cells to kill cancer cells.[23] Several studies showed that high amounts of circulating Tregs are negatively associated with OS in all PDAC stages.[12, 13] Another type of T cells with mainly regulatory functions in cancerous environments consists of gamma delta T cells (γδ T cells). This small subset of T cells has a different T cell receptor on their surface compared to the most common alpha beta T cells (αβ T cells). Although γδ T cells might have important immune-stimulating properties, such as antibody production by B cells, stimulation of αβ T cells and NK cells and maturation of DCs, they mainly act as γδ FoxP3+ Tregs in cancer, causing cancer cell proliferation and inhibition of the normal cytotoxic T cell response.[24] The prognostic role of circulating γδ T cells in PDAC patients still has to be evaluated.

Circulating cytotoxic T cells, also known as CD8+ T cells or effector T cells, should thus contribute to favorable outcomes, because they have a direct cytotoxic effect on cancer cells.[25] This indeed was found in patients with both resectable and advanced PDAC, in which high amounts of CD8+ cells in the peripheral blood are associated with a survival benefit.[13] Central memory T cells are a special subset of CD8+ T cells that respond faster and more efficient than normal CD8 + T cells, due to their previous encounters with cancer cells. The number of central memory T cells in the circulation was found to be an important factor for prognosis in resected PDAC patients.[26]

As the name suggests, helper T cells are a type of T cells that helps the activity of other immune cells. The major subgroups of Th cells are Th1, Th2, and Th17 cells with each distinctive functions. The main effector cells targeted by Th1 cells are macrophages and cytotoxic T cells, whereas the main effector cells of Th2 cells are B cells and granulocytes. Therefore, these Th cells act as pro-inflammatory, tumor-suppressing cells. On the other hand can Th17 cells produce the tumor-promoting cytokine IL-17 and stimulate Tregs, thus promoting cancer growth.[27]

Natural killer cells are cytotoxic cells as well, but in contrast to cytotoxic T cells, NK cells are not dependent on antigen presentation to initiate an immune reaction towards cancer cells. Cytokines such as IL-2 and IL-12 enhance NK cell activity.[28] In a cancerous environment, however, the killer function of NK cells is often impaired and the NK cell inhibitory receptor engagement by MHC class I molecules on tumor cells can prevent cytotoxicity efficiently.[28] In clinical studies, PDAC patients with diminished NK cell counts, representing a suppressed NK function, indeed had shorter OS and PFS.[10]

Lysis of cancer cells, induced by NK cells, leads to the release of small cancer cell particles that can be used by DCs as new tumor antigens. Dendritic cells will present these antigens to cytotoxic T cells, and thereby upregulate the immune response even further.[29] Although DCs represent only a small percentage of the total circulating immune cell count (approximately 1-2%), DC levels are correlated to OS[30] and long-term PDAC survivors show a relatively high amount of DCs in the peripheral blood.[17] Also, advanced stages of disease are associated with lower levels of DCs.[30] However, this prognostic favorable outcome is probably only true for mature antigen-presenting DCs. Tumor-derived factors can restrict DC maturation, leading to an increased level of immature DCs. These immature cells are still unable to present antigens and show more pro-tumor effects.[31] Immature DCs promote angiogenesis and tumor growth. If this effect is also visible in PDAC has to be investigated.

The prognostic value of monocytes in patients with PDAC is controversial. Monocytes are derived from immature myeloid cells and migrate from the bone marrow to inflamed tissues. They can differentiate into granulocytes, DCs, and macrophages.[32] These cells are important for the removal of cells and cell debris as well as for antigen presentation. Several studies have shown that an increase in the level of peripheral monocytes predicts poorer prognosis instead of survival benefit.[10, 11] An explanation might be found in macrophage polarization. While 'M1' macrophages are tumor suppressor cells with phagocytic and antigen-presenting features, 'M2' macrophages are tumor-promoting cells known as tumor-associated macrophages (TAMs). These M2 macrophages lose the ability to phagocyte cells and produce proangiogenic and anti-inflammatory cytokines, such as IL-10 and TGF-β.[20] The high number of monocytes in cancer patients might indicate an increased number of macrophages, though with a shift from the M1 to the M2 subtype. This shift results in a tumor-promoting instead of a tumor-suppressing environment.

In chronically inflamed and/or cancer environments, immature myeloid cells no longer differentiate into monocytes. Instead, these immature precursor cells migrate directly to the tissues where they act as immune suppressors, known as myeloid-derived suppressor cells (MDSCs). These induce immunosuppressive cells, cause impaired lymphocyte homing, and activate inhibitory immune checkpoint molecules.[33] For this reason, increased levels of MDSCs in patients with PDAC are associated with poor prognosis; the level of MDSCs could, therefore, be used to monitor progression of disease.[32, 34] Treatment with chemotherapy can turn the negative effect of MDSCs around, and diminish the amount of MDSCs.[32, 34]

IMMUNE CELL RATIOS

An immune cell ratio is a calculation using the absolute cell counts of at least two types of immune cells. Ratios can represent the balance between the 'good' and the 'bad' players of the immune response. A high neutrophil-to-lymphocyte ratio (NLR) in the peripheral blood is for that reason a bad prognostic factor for OS and PFS in all PDAC disease stages,[35, 36] because it represents an increase of 'bad' neutrophils and/or a decrease of 'good' lymphocytes. Similar results are found for the platelet-to lymphocyte ratio (PLR),[37] and the systemic immune-inflammation index (SII), calculated as the NLR multiplied by the platelet count,[38] in which platelets are the 'bad' key players. Platelets are known for their cancer-promoting effects besides hemostatic properties.[39] Platelets seem to have cancer-promoting properties influencing the majority of the classic hallmarks of cancer: platelets can activate pathways usually activated by oncogenic mutations and stimulate

cell proliferation, they can inhibit cell apoptosis, initiate the release of proangiogenic factors, and protect tumor cells from immune surveillance.[39, 40]

For the lymphocyte-to-monocyte ratio (LMR), the opposite effect holds true: an increase in poor prognostic monocytes gives a decrease in the LMR, leading to shorter OS and PFS in PDAC.[41] Alterations of the ratio between immune-suppressive CD4+ Tregs and immune-stimulating CD8+ cytotoxic T cells are of prognostic value as well. A relative increase of circulating Tregs compared to cytotoxic T cells is associated with disease progression and worse OS.[12]

CYTOKINES

Cytokines are small proteins (~20 kDa), produced by a wide variety of cells, and play an important role in the function and interaction of cells. Cytokines can be differentiated in chemokines, lymphokines, interleukins, interferons and tumor necrosis factors. Cytokines can have both pro-inflammatory and anti-inflammatory functions. Most cytokines are produced by immune cells, but normal epithelial cells, (cancer associated) fibroblasts, and cancer cells also produce interleukins, and other cytokines, enhancing their tumor-promoting effects. Some of these cytokines have immune-suppressive, tumor-promoting properties and are therefore poor prognostic factors and associated with advanced disease in PDAC patients. Others are immune-stimulatory and tumor-suppressive and are associated with a survival benefit. Cytokines known for their role in PDAC and their tumor-suppressive and tumor-promoting functions are summarized in Table 2. Cytokine functions may vary in different environments and cell types, their working mechanism is often ambiguous, and their function may rely on the concentration in the circulation or tissue.

Tumor-promoting cytokines mostly stimulate immune-suppressive components of the immune system. Interleukin-8, IL-6, and IL-18 are some of the most investigated cytokines in solid cancers, including PDAC.

Interleukin-6 is important for the maturation of B cells, but also necessary for other physiological functions, such as acute-phase protein synthesis and osteoclast production. Interleukin-6 is often dysregulated in cancer and involved in tumor promotion, cell proliferation, metastasis, and cell metabolism.[42] Interleukin-6 protects cancer cells from DNA damage induced by cancer treatment, oxidative stress and apoptosis.[42] The prognostic role of IL-6 in PDAC has been confirmed, including its association with advanced disease and progression of metastases,[43, 44] resulting in diminished OS and PFS.[45-47]

Table 2. Tumor-suppressive and tumor-promoting functions of cytokines involved in pancreatic cancer.

Cytokine	Tumor-suppressive functions	Tumor-promoting functions
IL-1β[48-50]	Production of pro-inflammatory cytokines Lymphocyte activation Proliferation of DCs, M1 macrophages, B cells Expansion of NK cells and cytotoxic T cells Apoptosis	Production of pro-inflammatory cytokines Production of IL-17 by γδ T cells Angiogenesis
IL-6[42]	Maturation of B cells Acute-phase protein synthesis Production of neutrophils	Production of pro-inflammatory cytokines, e.g. TNF-α and IL-1β Tumor promotion Cell proliferation Metastasis Production of neutrophils
IL-8[51]	Activation of the innate immune response Chemotaxis and activation of neutrophils Phagocytosis	Angiogenesis Tumor growth Metastasis
IL-10[52]	Activation and differentiation of mast cells, cytotoxic T cells, NK cells and B cells	Differentiation of Tregs Inhibition of pro-inflammatory cytokine production Suppression of antigen presentation Regulation of T cell and NK cell function
IL-11[53]	Acute-phase protein synthesis Production of platelets Apoptosis B cell differentiation and immunoglobulin synthesis	Inhibition of pro-inflammatory cytokine production
IL-12[54, 55]	Production of cytokines, e.g. IFN-γ and TNF-α by T cells and NK cells Stimulation of cytotoxic functions of T cells and NK cells Differentiation of naïve T cells into Th cells	
IL-17[32, 56]	Production of antimicrobial peptides Chemotaxis of neutrophils	Polarization of tumor-suppressing into tumor-promoting T cells Induction of MDSCs
IL-18[57, 58]	Production of IFN-γ Activation of NK cells Inhibition of angiogenesis Apoptosis	Production of IFN-γ and TNF-α, causing chronic inflammation Tumor growth Metastasis
IL-22[59]	Wound healing Production of antimicrobial molecules	Cancer cell survival and proliferation
IL-23[48, 54]	Production of Th17 cells Proliferation of memory T cells Promotion of antigen presentation by DCs	Production of γδ T cells
IL-29[60]	Production of pro-inflammatory cytokines, e.g. IL-6 and IL-8 Production of antiviral proteins Upregulation of MHC class I expression	Production of pro-inflammatory cytokines, causing chronic inflammation Tumor growth Metastasis
IL-33[61, 62]	Production of Th2 cells, mast cells, granulocytes Wound healing	Chronic inflammation Enhanced accumulation of mast cells, inducing tumor-promoting microenvironment

Table 2. Tumor-suppressive and tumor-promoting functions of cytokines involved in pancreatic cancer. (continued)

Cytokine	Tumor-suppressive functions	Tumor-promoting functions
IFN-γ[63]	Upregulation of class I and II antigen presentation Apoptosis Chemotaxis of leukocytes Differentiation of naïve T cells into Th1 cells	
TGF-β[64]	Production of Th17 cells Inhibition of cancer cell proliferation	Differentiation of Tregs Inhibition of differentiation of naïve T cells into Th1 cells Inhibition of B cell maturation Inhibition of immunoglobulin synthesis B cell apoptosis Suppression of macrophage functions Neutrophil differentiation into N2 phenotype
TNF-α[49, 65]	Apoptosis Phagocytosis Acute-phase protein synthesis Chemotaxis and activation of neutrophils	Tumor growth Angiogenesis Metastasis
VEGF[66]	Angiogenesis, causing accumulation of effector immune cells	Angiogenesis Accumulation of Tregs Tumor growth Metastasis Protection from cancer cell apoptosis

DC = dendritic cell, IFN = interferon, IL = interleukin, MDSC = myeloid-derived suppressor cell, MHC = major histocompatibility complex, NK = natural killer, TGF = transforming growth factor, Th = T helper, TNF = tumor necrosis factor, Treg = regulatory T cell, VEGF = vascular endothelial growth factor.

Under normal circumstances, IL-8 is a (neutrophil) chemotactic factor, which is important in acute inflammation. In cancer, however, neutrophils are involved in tumor-promoting processes, as discussed above. Moreover, IL-8 has proangiogenic effects, which promotes tumor growth.[48] High levels of circulating IL-8 are associated with cancer progression and metastasis, with higher levels in patients with metastatic disease, compared to locally advanced or resectable disease.[49] High levels of IL-8 have a negative effect on OS and PFS in all PDAC stages.[49, 50]

Several studies have clarified that other cytokines, such as IL-1β,[45, 50] IL-10,[47, 50] VEGF,[50] tumor necrosis factor-alpha (TNF-α),[50, 51] IL-29,[52] and IL-33[53] can also facilitate tumor growth, angiogenesis and metastasis, resulting in shorter OS and/or PFS. Moreover, different cytokines, such as IL-6, IL-1, and IL-17, stimulate the production of C-reactive protein (CRP),[54] an acute phase protein produced by hepatocytes and associated with infections and inflammation. It binds to damaged plasma membranes of cells and pathogenic organisms and consecutively activates the complement pathway. The complement cascade then leads to an increase of inflammatory cells and phagocytosis by macrophages.[55] Increased CRP levels in PDAC patients, suggesting chronic inflammation, are associated with poor OS.[56, 57]

Contrarily, various interleukins may correlate positively with survival. For one, the level of anti-inflammatory IL-11 in patients with metastatic disease is lower than that in patients with earlier disease stages, and is associated with better OS in patients of all stages with high levels.[58] A similar beneficial effect on survival has been found for IL-12[59] that is produced by DCs, and an increase of IL-12 might indirectly indicate an increase in DCs. Interleukin-12 also induces cytokine production, mainly interferon-gamma (IFN-γ), by NK and T cells and boosts their cytotoxic effects.[60]

One of the key players in the tumor-suppressive immune response is IFN-γ. This cytokine upregulates antigen presentation via major histocompatibility complex (MHC) class I and II, chemotaxis of leukocytes, and differentiation of Th1 cells.[61] Moreover, IFN-γ is an activator of macrophages and, more importantly, polarizes macrophages into the M1 tumor-suppressive subtype.[20, 61] Figure 1 presents an overview of the most important immune cells and cytokines balancing between a tumor-suppressive and tumor-promoting immune environment. In the overview, IFN-γ can be seen involved in many tumor-suppressive interactions between immune cells.

Apart from OS, response to therapy as well has been linked to cytokine levels.[62] For instance, a study found that levels of IL-23, IL-17 and TGF-β1 in responders to gemcitabine-based chemotherapy were lower than the corresponding levels in non-responder patients with progressive disease.[62] Moreover, IL-6, TGF-β1 and IL-23 may induce IL-17-producing, pro-inflammatory Th17 cells, which constitute a particular subset of T helper lymphocytes.[47, 62] Both high levels of TGF-β1 and IL-17 were found to be negative prognostic factors for OS in patients with PDAC.[62] Interleukin-17 has also been described as a cytokine secreted by gamma-delta T cells (γδ T cells), a tumor-infiltrating T cell subtype known for its capability to polarize tumor-suppressing T cells into tumor-promoting T cells.[63] The production of IL-17 by γδ T cells is induced by IL-1 and IL-23.[64] Another interleukin induced by IL-1β and IL-23 is IL-22. This interleukin is produced by several types of immune cells, but its receptor is only present on tissue cells, such as stromal and epithelial cells. IL-22 promotes cell survival and proliferation, indirectly supporting carcinogenesis.[65] PDAC is associated with higher IL-22 levels, but its prognostic value has not yet been described in the literature for this specific cancer.

In cancer environments TGF-β acts as the counterpart of IFN-γ, with mainly tumor-promoting immune system effects. Besides the activation of Th17 cells and Tregs, TGF-β is a pro-inflammatory cytokine produced by tumor-promoting M2 macrophages,[20] and causes polarization of N2 neutrophils.[19] Pancreatic cancer cells themselves can produce TGF-β as well.[20] The tumor-promoting role of TGF-β set off against the tumor-suppressive effects of IFN-γ is visualized in Figure 1.

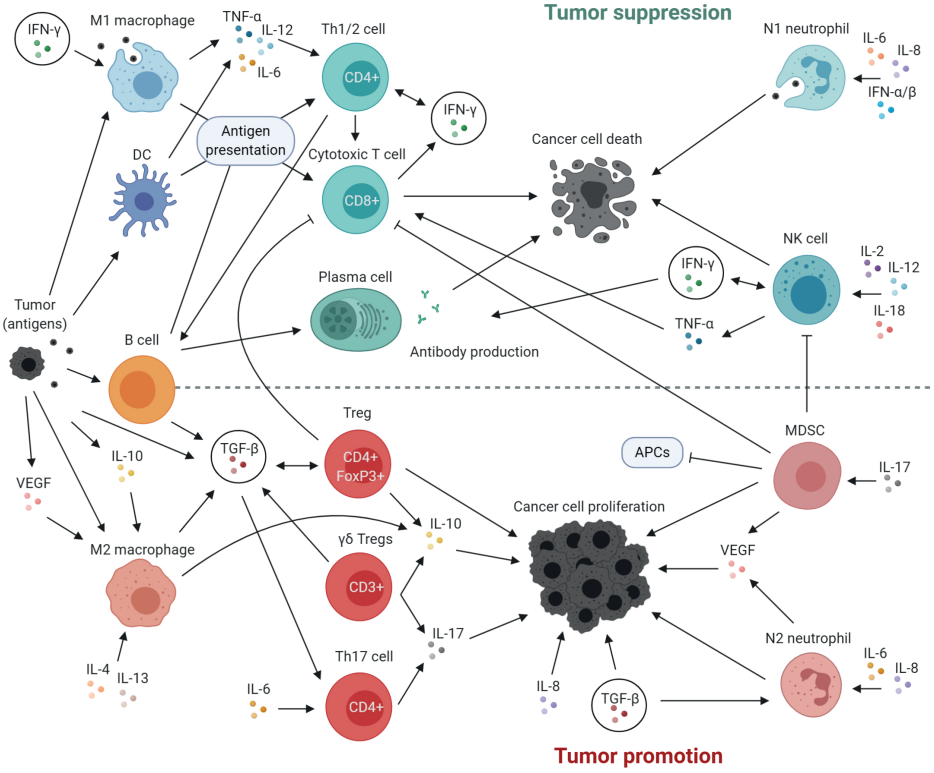

Figure 1. Schematic overview of the most important immunologic players and their interactions in tumor suppression and tumor promotion in pancreatic cancer.

The development and progression of cancer are tied to a fragile balance between tumor-suppressive and tumor-promoting functions of immune cells and cytokines. While tumor suppression is mainly IFN-γ-driven, tumor promotion is more dependent on TGF-β. IFN-γ polarizes macrophages towards the tumor suppressive M1 phenotype, promotes NK cells activity, and is important for Th1 cell differentiation and has a positive effect on antibody production by plasma cells. TGF-β is produced by cancer cells as well as by tumor-promoting M2 macrophages and leads to neutrophil polarization into a N2 phenotype. B cells can behave both tumor-suppressive as well as tumor-promoting. Also certain cytokines, such as IL-6, and IL-8, have ambiguous functions: they stimulate neutrophils which has a tumor suppressing effect in N1 and a tumor-promoting effect in N2 neutrophils. Moreover, IL-6 is a stimulator of T helper cells, of which Th1/2 are tumor-suppressive, and Th17 are mainly tumor-promoting. This figure is created with BioRender.com.

APC = antigen presenting cell, DC = dendritic cell, IFN = interferon, IL = interleukin, MDSC = myeloid-derived suppressor cell, NK = natural killer, TGF = transforming growth factor, Th = T helper, TNF = tumor necrosis factor, Treg = T regulatory cell, VEGF = vascular endothelial growth factor.

Another interleukin associated with response to therapy is IL-18. A study found that baseline serum levels of IL-18 in PDAC patients treated with gemcitabine chemotherapy were lower in patients with disease control than in patients with progressive disease.[66] The secretion of IL-18 at normal levels stimulates immunity and has antitumor effects via production of IFN-γ, activation of NK cells and initiation of apoptosis. However, higher secretion levels in PDAC patients have an opposite effect, causing chronic inflammation, thereby contributing to the pathogenesis of the disease.[66, 67] In addition to predicting

response to therapy, high levels of IL-18 are a negative predictor for advanced disease and OS.[66, 67] The exact mechanism of the altered function of IL-18 remains unknown.

IMMUNE CHECKPOINTS

Immune checkpoints are a cascade of signals that control the activation and function of immune cells. With the presentation of a peptide on MHC complexes by antigen-presenting cells, costimulatory signals are necessary to interact with the T cell receptor in order to activate T cells. Inhibitory immune checkpoints, on the other hand, are important to maintain self-tolerance, and to protect cells from autoimmune reactions. Binding of immune checkpoint co-stimulatory signals upon T cell receptor activation will prevent T cell activation and therefore T cell induced cell death. Pathogens usually lack these immune checkpoints and will therefore be attacked by a T cell response.[68] However, antigen presentation itself is also an inhibitory signal to cytotoxic T cells, by providing a negative feedback loop and thus preventing excessive cytotoxicity as a reaction to pathogens.[68] Cancer cells often express inhibitory immune checkpoint molecules, and inhibitory immune checkpoints are often hyperactivated in cancer environments,[69] diminishing the cytotoxic effects of T cells on cancer cells.[68] Some examples of inhibitory immune checkpoint molecules detected in PDAC are BTN3A1, PD-1, PD-L1, BTLA and pan-BTN3. High concentrations of inhibitory molecules in the circulation testify to the hyperactivation of the inhibitory pathways, whereby cancer cells can escape from cytotoxic immune effects. Therefore, upregulation and/or hyperactivation of inhibitory immune checkpoints, for example PD-1, are of prognostic significance in PDAC.[70] The same effect for the inhibitory immune checkpoint CTLA-4 was visible: CTLA-4 decreases the cytotoxic function of T cells and high CTLA-4 levels are associated with shorter OS.[47] The immune checkpoint TIM3, however, shows the opposite: higher levels of T cells expressing TIM3 were associated with increased survival, indicating its costimulatory role.[47] In Table 3 a summary of immune characteristics and their association with prognosis in PDAC patients is provided.

Overall, though, the detection of immune checkpoints in the circulation of PDAC patients and its implications is not well studied, but many attempts have taken place to investigate the therapeutic role of immune checkpoint inhibitors (ICI) in PDAC. Immune checkpoint inhibitors are antibodies that target immune checkpoints and block their function, so that cytotoxic activity of immune cells is not suppressed any longer, allowing for attack of cancer cells. Since ICI therapy resulted in a significant increase in survival in, for example, melanoma patients,[71] many research efforts are being directed to investigate the effect ICIs in other solid tumors. Examples of ICI therapy, used

Table 3. Summary of circulating immune characteristics associated with prognosis in patients with pancreatic cancer, according to current literature.

Poor prognosis in PDAC	Improved prognosis in PDAC
Cell types	
Neutrophils, total population	Lymphocytes, total population
B cells	Effector T cells
Regulatory T cells	T helper 1/2 cells
T helper 17 cells	Memory T cells
Monocytes, total population	Natural killer cells
Myeloid-derived suppressor cells	Dendritic cells
Platelets	
Immune cell ratios	
Neutrophil-to-lymphocyte ratio	Lymphocyte-to-monocyte ratio
Platelet-to-lymphocyte ratio	
Systemic immune inflammation index	
CD4+/CD8+ ratio	
Cytokines	
IL-1β, IL-6, IL-8, IL-10, IL-17, IL-18, IL-22, IL-23, IL-29, IL-33, TGF-β, TNF-α, VEGF	IFN-γ, IL-11, IL-12
Other	
C-reactive protein	
Inhibitory immune checkpoint molecules	

IFN = interferon, IL = interleukin, PDAC = pancreatic ductal adenocarcinoma, TGF = transforming growth factor, TNF = tumor necrosis factor, VEGF = vascular endothelial growth factor.

in pancreatic cancer, are anti-CTLA-4 (e.g. ipilimumab), anti-PD1 (e.g. nivolumab and prembrolizumab), and anti-PD-L1 (e.g. atezolizumab and avelumab). Unfortunately, ICI monotherapy has currently little effect in PDAC patients, but survival benefit might occur when combining ICIs with chemotherapy or radiation.[72, 73] One of the commonly accepted explanations for ICI therapy failure is the imbalance in T cell activity with diminished amounts of cytotoxic T cells and an increased amount of Tregs. This way, the target for ICIs is already lacking.[74]

FUTURE THERAPIES

The question remains how other immune signatures can be used to develop new therapies, besides immune checkpoint blockage. One possible option is to stimulate pro-inflammatory, tumor-suppressing immune cells to increase their numbers and activity. Previous studies have already shown promising results on expansion and endogenous activation of NK cells,[28] and DCs.[75] T cells can be genetically engineered to potent their

cytotoxic activity against specific cancer-associated antigens with the use of chimeric antigen receptor (CAR) T cell therapy for PDAC.[76] Increasing evidence shows that direct stimulation or inhibition of cytokines could improve survival in PDAC. In mouse models, the use of monoclonal antibodies against the IL-17B receptor prevented metastasis of pancreatic cancer cells and resulted in longer survival.[77] High dose IL-2 has possibly a direct cytotoxic effect on cancer cells, and may therefore increase survival in patients.[78] Nevertheless, the majority of trials on immune cell stimulation and cytokine therapy are still in the preclinical phase. Besides, when aimed at quite unselective targets, immune therapy in general is often accompanied by severe side effects.[78]

At this moment, treatment with one or more ICIs, most times combined with forms of radiation therapy, is the most investigated type of immune therapy according to the ClinicalTrials.gov registration, even though current results are disappointing. This is followed by vaccine therapy, which comprehends any kind of immune stimulatory substances, including inactivated tumor cells, antigens/peptides, oncolytic viruses, and activated DCs.

CONCLUSION

Multiple components of the immune system in the peripheral blood have been found to be of prognostic significance in PDAC patients. However, the function of immune cells is ambiguous with often both tumor-suppressive as well as tumor-promoting effects, and immune markers cannot yet be implemented as a biomarker in clinical practice. Further clinical studies are required to investigate the complexity of key players in the immune system of which the results could be the foundation for future therapies.

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Chapter 9

RNA from stabilized whole blood enables more comprehensive immune gene expression profiling compared to RNA from peripheral blood mononuclear cells

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ABSTRACT

Monitoring changes in the immune profile in blood samples can help identify changes in tumor biology and therapy responsiveness over time. Immune-related gene expression profiles offer a highly reproducible method to monitor changes of the immune system. However, measuring gene expression profiles in whole blood samples can be complicated because of the high protein and enzyme abundancy that affect the stability and quality of the RNA. Peripheral blood mononuclear cells (PBMCs) are one the most commonly used sources for immune cell RNA extraction, though, this method does not reflect all components of the peripheral blood. The aim of this study was to determine the differences in immune-related gene expression between RNA isolated from stabilized whole blood and RNA isolated from PBMCs. Whole blood samples from twelve pancreatic cancer patients were collected before and after chemotherapy (n=24). Blood samples were collected in both EDTA tubes, and Tempus tubes containing an RNA stabilizer (total n=48). PBMCs were isolated from EDTA samples using Ficoll and were snap frozen. Subsequently, immune-related gene expression was profiled using the PanCancer Immune Profiling Panel of NanoString technology. Gene expression profiles of PBMCs were compared to that of Tempus tubes using the Advanced Analysis module of nSolver software. Both types of samples provided good quality RNA and gene expression measurements. However, RNA isolated from Tempus tubes resulted in significantly higher gene counts than PBMCs; 107/730 genes were exclusively detected in Tempus samples, while under the detection limit in PBMCs. In addition, 192/730 genes showed significantly higher gene counts in Tempus samples, 157/730 genes showed higher gene counts in PBMCs. Thus, RNA isolated from whole blood stabilizing blood tubes, such as Tempus tubes, enable higher gene counts and more comprehensive measurements of gene expression profiles compared to RNA isolated from PBMCs.

INTRODUCTION

In this era of rapidly evolving immune monitoring and immune therapies in many different research fields, longitudinal peripheral blood immune profiling is becoming crucial to monitor the outcome of patients.[1] Peripheral blood sampling is a low-risk, non-invasive procedure and available at low costs.[2] Peripheral blood is the main route for transportation of immune cells, which thereby provides the opportunity to monitor the activity of the immune system.[3,4] Monitoring the immune system requires more than profiling subtypes of immune cells. Blood-based gene expression profiles reflect activity of the immune response, including all types of circulating immune cells, their secreted cytokines, chemokines, and neoantigens.

Peripheral blood mononuclear cells (PBMCs) are one of the most commonly used sources of RNA to define the immune response in research.[5] Isolation of PBMCs provides a pellet of immune cells which can be stored after snap freezing to preserve the RNA. However, this pure mononuclear cell pellet mainly consists of lymphocytes,[6] while other immune cells, such as erythrocytes, platelets, and granulocytes are completely washed out.[7] All circulating RNA and secreted factors are lost during the various washing steps to purify PBMCs. Additionally, PBMC isolation is a time-consuming procedure and the method varies between laboratories.[8] On the other hand, whole blood represents the complete range of immune cells and their secreted factors and is easy to obtain. However, whole blood contains several proteins, enzymes, and RNases that prevent reliable isolation of RNA.[9] Collecting whole blood in tubes containing an RNA stabilizing reagent could provide a solution to this undesired phenomenon.[10,11] Using an RNA stabilizer enables isolation of RNA from all blood components and secreted factors, instead of mononuclear cells only. In addition, such samples capture expression profiles that accurately reflect the transcriptome at time of blood collection with minimum sample handling artifacts. However, blood samples will be diluted with the stabilizing reagent and the percentage of RNA isolated from lymphocytes might be lower compared to PBMC pellets.

In this study, we compared the abundancy of measured immune-related genes, sensitivity of detection and the range of identified immune cells between RNA isolated from PBMCs and RNA isolated from whole blood samples collected in Tempus tubes containing a stabilizing reagent.

MATERIALS AND METHODS

Patient selection

Samples from twelve patients, participating in a prospective cohort study including repeated peripheral blood sampling, were used in this experiment. All patients were diagnosed with pancreatic cancer and treated with FOLFIRINOX chemotherapy. Blood samples were collected the day before start of treatment and after one cycle of chemotherapy. Both blood samples for PBMC extraction in EDTA tubes (*n*=24) and whole blood samples in Tempus tubes (*n*=24) and were collected within the same blood draw at each time point resulting in a total *n*=48.

This study was approved by the medical ethics committee of the Erasmus University Medical Center Rotterdam (MEC-2018-087) and samples were collected with patients' written informed consent.

Blood sample processing

For isolation of PBMCs, whole blood was collected in 10 mL EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Within a maximum of four hours after collection, PBMCs were extracted with Ficoll-Paque technique, using LeucoSep tubes (Greiner Bio-One, Kremsmünster, Austria). After isolation and cell count with the Countess II Automated Cell Counter (Invitrogen, Carlsbad, CA, USA), PBMCs were snap frozen and stored at -80˚C until further use. On average, 4 mL of whole blood was used to store one vial of PBMCs.

Whole blood was collected in Tempus tubes (Applied Biosystems, Foster City, CA, USA), in which 3 mL of whole blood is stabilized with 6 mL of RNA stabilizing reagent, resulting in a total volume of 9 mL. Tubes were stored at -80˚C within four hours after collection until further use.

RNA isolation

Total RNA from PBMCs was isolated from approximately $8.4x10^5$–$1.3x10^7$ cells using the RNeasy Mini isolation kit (Qiagen, Hilden, Germany). Total RNA from Tempus tubes was isolated from 400 µL of stabilized whole blood using the NucleoSpin RNA Blood isolation kit (Macherey-Nagel, Düren, Germany). If concentrations were not sufficient, RNA was again isolated from 800 µL of stabilized whole blood instead. RNA concentrations and quality were measured using the Agilent 2100 BioAnalyzer (Santa Clara, CA, USA). To correct for degradation of the RNA, the percentage of fragments of 300–4000 nucleotides was used to calculate the corrected concentrations.

Gene expression analysis

Gene expression analysis was performed using the PanCancer Immune Profiling Panel from NanoString technologies. The panel contains probes of 730 immune-related genes and 40 housekeeping genes, representing 24 different immune cell types and common checkpoint inhibitors, covering both the adaptive and innate immune response. For each sample, 200 ng of total RNA, with a maximum of 7 µL (>28.6 ng/µL), was used. Hybridization was performed at 65˚C for 17 hours using a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The nCounter Flex system (NanoString, Seattle, WA, USA) was used for sample preparation. Gene counting was performed by scanning 490 Fields of View.

Statistical analysis

Gene expression profiles of RNA isolated from PBMCs collected in EDTA tubes were compared with RNA isolated from Tempus tubes. Raw gene counts were normalized using the most stable housekeeping genes from the panel selected by the geNorm algorithm,[12] incorporated in the Advanced Analysis module of nSolver software (version 2.0, NanoString, Seattle, WA, USA). Background threshold was determined as the average count of the negative controls +2 standard deviations. Genes that showed a count above the threshold were considered as detected genes. Gene counts of most genes were not normally distributed across samples, even after logarithmic transformation. Therefore differential expression of genes between PBMC and Tempus RNA was tested with Mann-Whitney U tests and Benjamin-Hochberg procedures were used to correct for multiple testing.

Differentially expressed (DE) genes were further analyzed with Gene Set Analysis (GSA) as part of the Advanced Analysis. It enables to connect DE genes based on their function, resulting in scores for various pathways and cell types. Pathways and cell types are predefined using specific gene sets, as described previously.[13] The definition of cell types using these gene sets were found to be strongly correlated to cell types measured by flow cytometry in different tissue types, including cancers.[14-16] Differences in pathway scores and cell type scores were tested with Mann-Whitney U tests and Benjamin-Hochberg procedures were used to correct for multiple testing.

Adjusted *P*-values <0.05 were considered statistically significant. Statistical analyses were performed with R software (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria), using the packages stats (version 3.6.1) and matrixStats (version 0.56.0).

RESULTS

RNA quality

All 24 PBMC samples isolated from EDTA tubes yielded sufficient RNA (>28.6 ng/µL). Ten of the Tempus samples showed insufficient RNA yields and required another isolation using 800 µL whole blood. After a second isolation, all Tempus samples yielded sufficient RNA concentrations. Also after correction of the RNA concentration for RNA with length of 300–4000 nucleotides, all samples showed good enough quantity and quality for immune gene expression profile measurements.

Gene expression

In total, 31 housekeeping genes were selected for normalization. An overview of these selected housekeeping genes with their average expression and variance can be found in Supplementary Table 1.

After normalization, the average gene count in PBMC samples was 790.02 and in Tempus samples 1188.64 ($P<0.001$). Out of 730 genes, 108 were not detected in any group (PBMCs or Tempus); the median count of these genes was below the threshold of 25 counts, as determined by the negative controls. A total of 107 genes was exclusively detected in Tempus samples. In contrast, only six genes were exclusively detected in PBMCs samples. Moreover, 157 genes were significantly higher expressed in PBMC samples with $P<0.05$ (of which 72 genes with a fold of change ≥2), while 192 genes were significantly higher expressed in Tempus samples with $P<0.05$ (of which 111 genes with a fold of change ≥2). A comprehensive flowchart can be found in Figure 1. The variation between PBMCs and Tempus samples per patient is visualized in Supplementary Figure 1. 21/24 patients show the same trend as in the total group: the number of detectable genes and the number of exclusively detectable genes were higher in Tempus samples compared to PBMC samples.

Unsupervised clustering for all genes resulted in a perfect clustering of PBMC samples and Tempus samples (Figure 2), highlighting that the preservation method affects the expression of immune-related genes more than the biological variation between samples.

Pathway scores

All pathway scores were significantly different between PBMCs and Tempus samples (Figure 3, Table 1). The pathways Antigen Processing ($P<0.001$), B cell Functions ($P<0.001$), Cell Cycle ($P<0.001$), Cytotoxicity ($P=0.014$), NK cell Functions ($P<0.001$), and Senescence ($P<0.001$) scored higher in PBMCs, while all other pathways scored higher in Tempus samples with $P<0.001$ (Table 1). Similar to the results of gene expression, PBMCs and

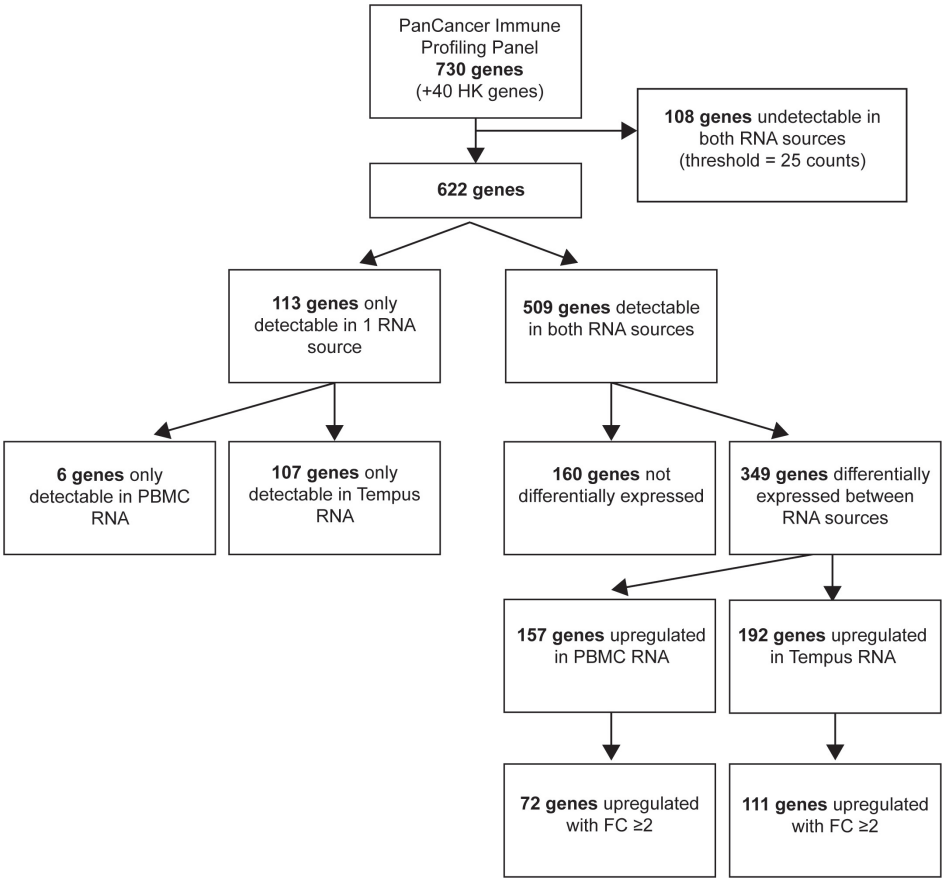

Figure 1. Diagram of genes detected or upregulated in one source of input RNA. Out of 700 detected genes, 107 were only detected in Tempus samples, six genes were only detected in PBMC samples. Most genes were upregulated in Tempus samples (192 genes), while 157 genes were upregulated in PBMC samples. HK = housekeeping.

Tempus samples clustered perfectly based on pathway scoring (Supplementary Figure 2). However, some pathway scores are based on genes that were hardly detected in PBMC samples. For example, the Cancer/Testis Antigen (CT Antigen) pathway is defined by twelve genes of which only one gene was detected in PBMC samples but eleven were detected in Tempus samples. In addition, Complement pathway is defined by fifteen genes, all of them were detected in Tempus samples, but only twelve were detected in PBMC samples. For the Interleukin pathway, the majority of genes, 25/38, was not detected in PBMCs. Also in the Chemokine pathway almost half of the defining genes, 42 out of 99, could not be detected in PBMC samples. In all pathways, less defining genes were detected in PBMC samples compared to Tempus samples. As presented in Supplementary Figure 1, in all samples more pathways are overexpressed in Tempus than there are pathways overexpressed in PBMC samples.

Table 1. Comparison of relative pathway scores between peripheral blood mononuclear cells (PBMCs) and Tempus samples.

Pathway	Score PBMCs, median (IQR)	Score Tempus, median (IQR)	Adjusted *P*
Adhesion	-1.85 (-2.97 - -0.70)	2.02 (0.09 - 3.16)	<0.001
Antigen Processing	1.10 (0.35 - 2.19)	-1.42 (-2.26 - -0.64)	<0.001
B cell Functions	1.99 (1.18 - 2.34)	-1.87 (-2.64 - -1.22)	<0.001
Cell Cycle	1.70 (0.77 - 2.05)	-1.39 (-1.59 - -1.18)	<0.001
Cell Functions	-3.16 (-3.84 - -2.20)	3.17 (1.16 - 4.17)	<0.001
Chemokines	-5.15 (-5.78 - -3.55)	4.27 (2.14 - 6.22)	<0.001
Complement[a]	-1.88 (-2.71 - -1.04)	1.17 (0.65 - 2.59)	<0.001
CT Antigen[a]	-3.50 (-4.62 - -0.89)	2.32 (1.35 - 4.31)	<0.001
Cytokines	-3.78 (-4.28 - -2.39)	2.96 (1.38 - 4.41)	<0.001
Cytotoxicity	0.76 (0.09 - 1.12)	-0.34 (-1.44 - 0.46)	0.014
Interleukins	-3.25 (-4.16 - -1.50)	2.12 (1.24 - 4.20)	<0.001
Leukocyte Functions	-1.13 (-1.98 - -0.21)	1.12 (0.06 - 1.78)	<0.001
Macrophage Functions	-1.41 (-1.80 - -0.83)	1.34 (0.68 - 1.98)	<0.001
Microglial Functions	-0.90 (-1.18 - -0.58)	0.95 (0.66 - 1.40)	<0.001
NK Cell Functions	1.56 (0.74 - 2.30)	-1.70 (-2.62 - 0.12)	<0.001
Pathogen Defense	-1.59 (-1.94 - -0.77)	1.25 (0.20 - 2.50)	<0.001
Regulation	-5.32 (-5.96 - -4.16)	5.27 (3.00 - 6.82)	<0.001
Senescence	1.25 (1.03 - 1.50)	-1.28 (-1.46 - 1.15)	<0.001
T cell Functions	-3.23 (-4.05 - -2.31)	3.42 (1.52 - 4.70)	<0.001
TLR[a]	-1.53 (-2.18 - -1.07)	1.98 (0.69 - 2.67)	<0.001
TNF Superfamily	-2.33 (-2.87 - -1.96)	2.50 (1.99 - 2.90)	<0.001
Transporter Functions	-1.83 (-2.21 - -1.31)	1.73 (1.18 - 2.28)	<0.001

P-values are calculated with Mann-Whitney U tests and adjusted with Benjamin-Hochberg procedure. IQR = interquartile range, TLR = toll-like receptor, CT antigen = cancer/testis antigen, NK = natural killer, TNF = tumor necrosis factor. [a]Defining genes were not detected in (all) PBMC samples.

Cell type scores

Expression of gene sets that are only expressed by one immune cell type were used to create a cell type score (Supplementary Table 2). The average cell type score was compared between PBMCs and Tempus samples (Table 2). The following cell types were found to be relatively higher in Tempus preservation methods: dendritic cells ($P<0.001$), CD45+ cells ($P<0.001$), mast cells ($P<0.001$), neutrophils ($P<0.001$), natural killer cells ($P=0.022$). However, cytotoxic cells ($P=0.024$), macrophages ($P=0.009$), B cells ($P<0.001$), and CD8+ T cells ($P<0.001$) were relatively higher in PBMC samples. Relative expression of cell types is visualized in Figure 4.

The definition of dendritic cells is based on the expression of three genes that all showed a median count below the negative threshold in PBMC samples. In Tempus samples, two

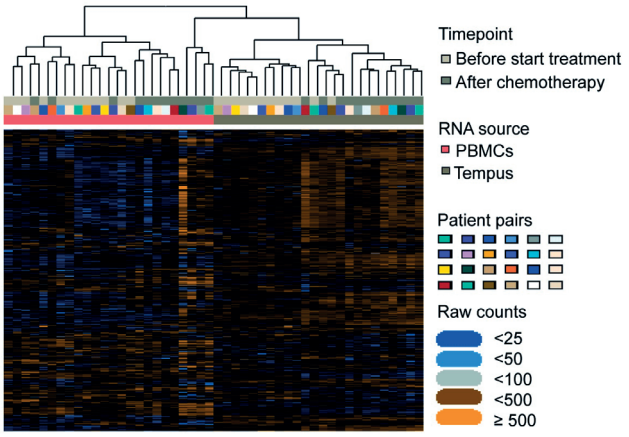

Figure 2. Heatmap of normalized data. Unsupervised clustering of PBMCs and Tempus samples resulted in a perfect clustering based on the sample type.

Table 2. Comparison of cell scores between peripheral blood mononuclear cells (PBMCs) and Tempus samples

Cell type	Score PBMCs, median (IQR)	Score Tempus, median (IQR)	Adjusted *P*
NK CD56dim cells[a]	5.71 (5.25-6.27)	5.61 (5.26-6.10)	0.633
Cytotoxic cells	9.54 (9.27-9.86)	9.14 (8.39-9.44)	0.024
Dendritic cells[a]	3.54 (3.34-3.89)	4.65 (4.14-5.06)	<0.001
Regulatory T cells	5.08 (4.65-5.75)	5.06 (4.74-5.50)	0.675
CD45+ cells	10.69 (10.48-11.14)	13.26 (12.93-13.71)	<0.001
Macrophages	9.10 (8.58-9.43)	8.53 (8.34-8.70)	0.009
T cells	8.11 (7.97-8.76)	7.95 (7.55-8.41)	0.136
Mast cells	4.47 (4.16-5.18)	5.81 (5.51-6.05)	<0.001
Neutrophils	11.85 (11.12-12.46)	14.38 (12.82-15.09)	<0.001
Natural killer cells	6.52 (6.30-7.34)	7.28 (6.79-7.44)	0.022
Exhausted CD8+ cells	6.40 (6.02-6.61)	6.36 (6.13-6.71)	0.633
B cells	7.72 (7.11-8.33)	6.64 (6.37-6.92)	<0.001
Th1 cells	8.05 (7.64-8.48)	8.19 (7.81-5.78)	0.548
CD8+ T cells	7.96 (7.34-8.40)	6.55 (6.12-7.07)	<0.001

Data are presented as log2 values. *P*-values are calculated with Mann-Whitney U tests and adjusted with Benjamin-Hochberg procedure. IQR = interquartile range, Th = T-helper. [a]Defining genes were not detected in (all) PBMC samples.

of the genes showed an insufficient median gene count below the threshold. For mast cells, one out of two defining genes was not detected in PBMC samples, while detectable in Tempus samples.

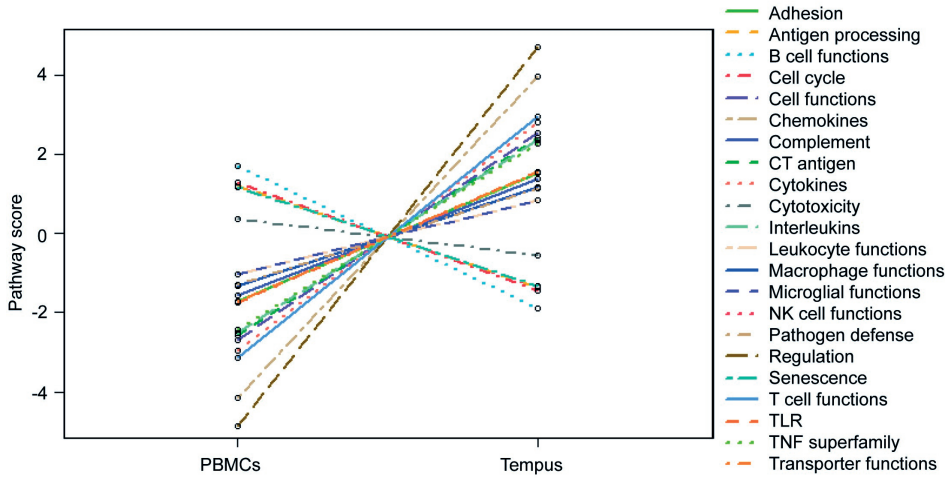

Figure 3. Relative expression of pathway scores between PBMCs and Tempus RNA. Six pathways (Antigen Processing, B cell functions, Cell Cycle, Cytotoxicity, NK cell functions, and Senesence) are upregulated in PBMC samples compared to Tempus samples. All other pathways (22) are upregulated in Tempus, reflecting the higher expression of detected genes in Tempus samples compared to PBMCs.

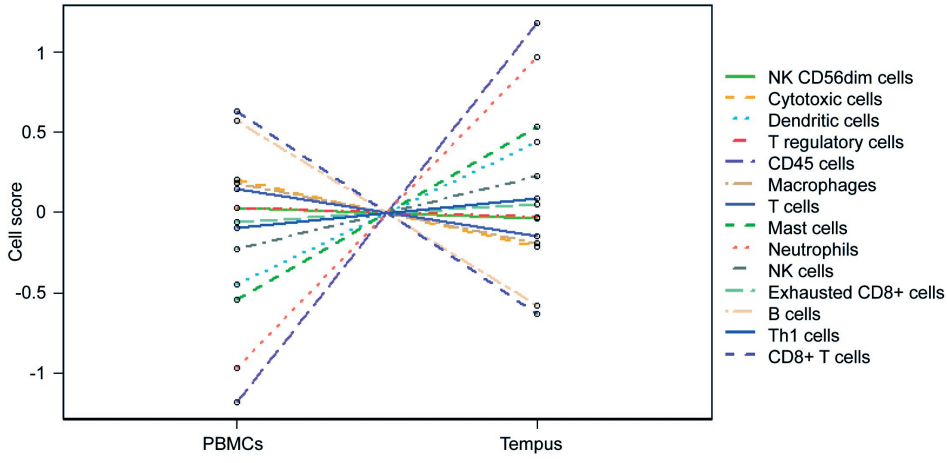

Figure 4. Relative expression of cell types between PBMCs and Tempus RNA. Dendritic cells, CD45+ cells, mast cells, neutrophils, and natural killer (NK) cells show higher scores in Tempus samples. Cytotoxic cells, macrophages, B cells and CD8+ T cells were detected at a higher level in PBMCs.

In 8/24 samples more cell types were overexpressed in Tempus. In 16/24 samples more cell types were overexpressed in PBMCs (Supplementary Figure 1).

Table 3. Pros and cons of using RNA from PBMCs or Tempus using NanoString Immunology gene expression analysis.

Pros PBMC RNA
Enriched RNA from mononuclear cells
Cons PBMC RNA
At least 4 mL of whole blood necessary Relatively long hands-on time for cell extraction until freezing of cells Important immunology related genes might not be detected due to the source of RNA (not mononuclear cells) Variability of isolation methods in different laboratories
Pros Tempus RNA
Only 3 mL of whole blood necessary, which can be used for multiple RNA extractions. On average, only 800 µL of stabilized whole blood (=267 µL of whole blood + 533 µL of stabilizing reagent) is needed per extraction. No cell extraction. Therefore, no hands- on time needed to process the samples. RNA preservation with stabilizing reagent RNA from all blood components and free RNA are included. Higher number of genes detected Higher average gene count
Cons Tempus RNA
Relatively expensive collection tubes (€7.06/tube) Dilution of immune cells and their RNA by stabilizing reagent Multiple RNA isolations might be necessary to reach the desired concentration of RNA

DISCUSSION

The aim of this study was to determine the most reliable method of RNA blood cell extraction for monitoring of the immune system by immune-related gene expression analysis. Although both RNA preservation methods resulted in good quality and quantity of RNA, gene expression profiles revealed a significant variation between PBMCs and Tempus samples. Nevertheless, gene expression profiles of both sample types showed promising results with a sufficient number of detected genes. To our knowledge, this is the first study to compare gene expression profiles of RNA isolated from PBMCs and RNA isolated from whole blood stabilizing tubes using NanoString technology.

The use of an RNA stabilizing reagent preserves gene activity and enables detection of a higher number of immune-related genes from whole blood samples compared to PBMC RNA. A total of 127 genes was detected exclusively in Tempus samples, while only six genes were detected exclusively in PBMC samples. Overall, average gene counts were 51% higher in Tempus samples compared to PBMCs. Higher gene counts enable a more reliable and comprehensive analysis, because the genes are certainly above the threshold. Using an RNA stabilizing reagent ensures that RNA from all sources is preserved, including RNA from all blood components and secreted RNA that is washed away when purifying PBMCs from EDTA tubes. For example, granulocytes are preserved in Tempus tubes, therefore the genes that define them showed higher counts in Tempus compared

to PBMCs (Table 2). In addition, the time consumed between drawing blood samples from patients, isolation of PBMCs, and storage at -80˚C is significantly longer (1–3 hours) than drawing blood in Tempus tubes that do not need any processing before storage. During blood processing and PBMC isolation, RNA is exposed to various enzymes resulting in RNA degradation, affecting the most vulnerable and low abundant dendritic cells first.[17] As a result, not only genes from non-mononuclear cells, but also genes associated with mononuclear cells, for example dendritic cells and natural killer cells, were measured at higher counts in Tempus samples compared to PBMCs. We hypothesized that enrichment of mononuclear cells in PBMCs would lead to detection of genes expressed by mononuclear cells at a higher level. However, in this study only a minority of genes was expressed at a higher level in PBMCs. On the other hand, blood collected in Tempus tubes is diluted with a stabilizing reagent, which might cause lower gene expression results, hence the relative higher counts of 157 genes in PBMCs compared to Tempus samples.

Unsupervised clustering showed perfect clustering of PBMC and Tempus samples, highlighting that the RNA preservation method should be chosen carefully when profiling RNA of blood samples. Therefore, measuring the gene expression profiles of RNA samples isolated from various blood tubes or sources in one experiment is not recommended. Although PBMCs and whole blood stored in Tempus tubes showed similar gene expression profiles, absolute gene expression levels did significantly differ, which was also visualized by clustering of samples by the type of RNA source. Similar results were found by Rollins *et al.* using Affymetrix arrays.[18]

As a consequence of the detection and gene count variation between PBMCs and Tempus samples, unsupervised clustering based on the pathway scores showed a perfect separation between the samples. Most pathways were upregulated in Tempus tubes compared to PBMCs, which is explained by the higher cell counts of all defined pathways. The average count of genes would not affect the pathway scores when samples of the same RNA source will be used in one experiment. The higher gene counts and therefore the scores of pathways are influenced by preservation of RNA with the stabilizing reagent. In addition, some pathway scores are based on genes that were not detected in PBMC samples. For example, the CT Antigen pathway is defined by twelve genes, of which only one gene was detected in the majority of PBMC samples. However, all twelve genes were detected in Tempus samples, resulting in a higher score of the CT Antigen pathway in Tempus tubes. Another example is the Complement pathway, which is defined by fifteen genes. All fifteen were detected in Tempus samples, while only twelve genes were detected in PBMCs samples, which reflects the higher score for Complement pathway in Tempus samples. At least the CT Antigen pathway should

be excluded from analysis in experiments when PBMC samples are used, because it is impossible to determine if the genes are not present or if they are under the detection limit. As another example, nine genes (out of eleven) defined as the TLR pathway were detected in all PBMC and Tempus samples, which should be sufficient to accurately score the pathway.

Granulocytes (neutrophils, mast cells) were not the only cells upregulated in Tempus tubes, but also mononuclear cells (e.g. dendritic cells), for which was enriched in PBMC samples. However, dendritic cell genes were all under the detection limit in PBMC samples. For mast cells only one out of two genes could be detected in PBMCs. In order to detect genes defining dendritic cells or mast cells, an RNA stabilizing reagent is needed to preserve the activity of these genes. Dendritic cells are known to represent only a very small percentage of the total blood cell count in the circulation.[6, 17] Their genes are vulnerable and could probably therefore not be detected in PBMCs. The definition of cells in nSolver software is limited by the number of genes used to define the cells. T helper 1 (Th1), regulatory T cells, and CD45+ cells are all based on the expression of one gene, therefore, the scores of these cell types needs to be confirmed. The other types of cells that were defined using multiple genes are more reliable (Supplementary Table 2). Quality controlling the data used in cell type definition and pathway scoring is extremely important in any experiment using NanoString technology. The fact that CD45+ cells are detected at a higher cell count in Tempus compared to PBMCs, even though in Tempus relatively less CD45+ cells are present and thus less RNA expression of the CD45 gene *PTPRC* is present, confirms our conclusion that RNA is preserved better in Tempus.

Besides the better RNA preservation, there are additional advantages of using stabilized whole blood samples, among which is the possibility to detect non-mononuclear cells. The immune system is not dependent on mononuclear cells alone, and other cells, such as granulocytes, are extremely important to investigate. The quantity and function of immune cells, including mononuclear cells, often depends on the presence and interaction with other types of immune cells. However, there are advantages and disadvantages for both sources of RNA for measuring immune-related gene expression. Collecting whole blood in RNA stabilizing tubes is convenient and does not require extra processing before storage. PBMC extraction from EDTA tubes, however, is a time-consuming method (±1 hour when using LeucoSep tubes for easy separation of PBMCs), and isolation protocols vary between researchers and laboratories. To reach sufficient expression data, PBMCs isolation from at least 4 mL of whole blood is necessary for RNA extraction. In Tempus tubes, extra RNA extractions using a higher amount, of input material (800 µL instead of 400 µL) was in this experiment necessary in 10/24 samples to reach sufficient RNA yields to perform multiplex measurements. However, this resulted in a maximum

of 400 µL undiluted whole blood used. An overview of pros and cons are summarized in Table 3. Whether the type of RNA preservation tube (e.g. Tempus versus PAXgene) affects RNA quality and expression is not clear yet. There is evidence that differences between these tubes have influence on absolute gene expression levels, but this effect might be overcome by modification of the isolation protocol.[4, 19]

Although not included in this experiment, differences in measured gene expression between RNA sources are probably not limited to NanoString technologies, but should also be considered in other types of gene expression analysis.

In conclusion, RNA isolated from PBMCs extracted from EDTA tubes and RNA isolated from Tempus tubes resulted in good RNA quality and quantity that enabled gene expression profiling with NanoString technology. Gene expression data were mostly affected by the RNA preservation method. Although profiling immune-related genes using RNA isolated from PBMCs provided sufficient gene counts and measurements, using RNA from stabilized whole blood, in this case collected in Tempus tubes, resulted in higher gene counts and thus more comprehensive measurements. Therefore, we do not recommend combining various sample types within the same experiment. In particular, certain cell types and pathways (dendritic cells, and Complement pathway) cannot be reliably measured when using PBMC samples.

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SUPPLEMENTARY FILES

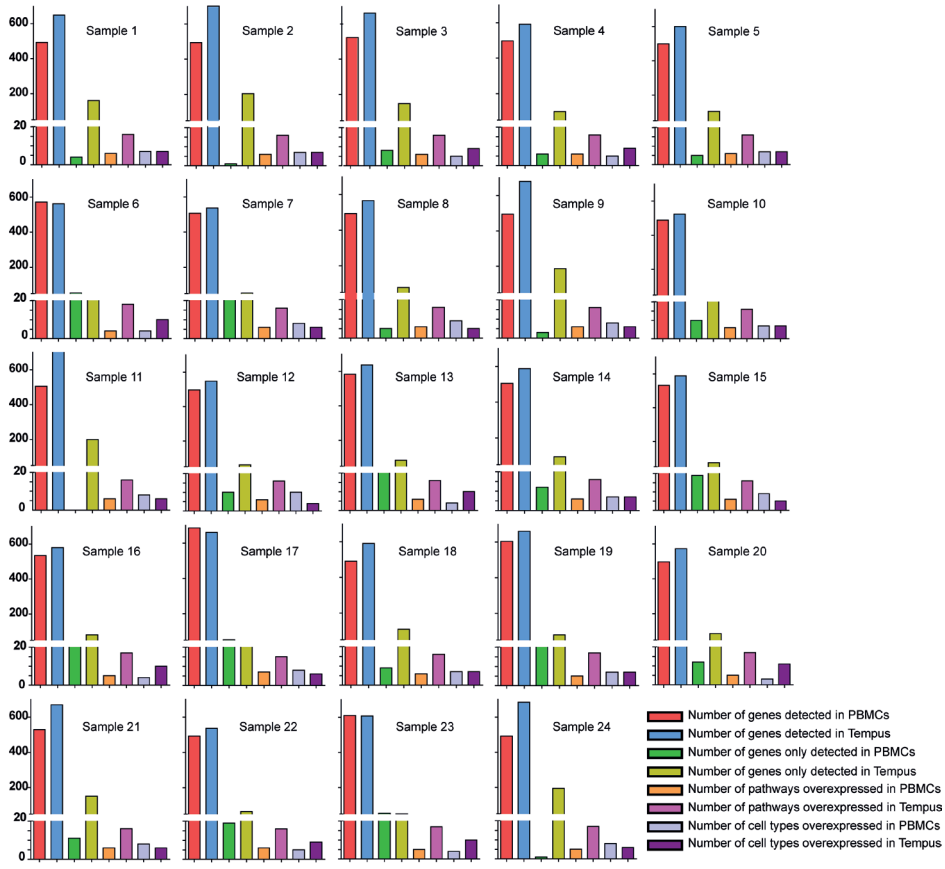
Supplementary Table 1. Housekeeping genes selected for normalization, with their average expression and variance.

Gene	Mean expression (Log2)	Variance (Log2)
TBP	7.91	0.03
EIF2B4	7.70	0.03
PRPF38A	9.01	0.03
HDAC3	7.78	0.04
ABCF1	7.93	0.09
MRPS5	8.82	0.09
SF3A3	8.43	0.15
DNAJC14	6.59	0.12
EDC3	7.57	0.18
SDHA	7.76	0.15
COG7	7.24	0.16
ERCC3	6.84	0.17
TLK2	8.28	0.16
CNOT4	7.81	0.14
NOL7	8.20	0.09
DHX16	7.16	0.13
AGK	6.74	0.11
DDX50	7.39	0.13
HPRT1	8.26	0.11
ZC3H14	6.95	0.12
PPIA	7.34	0.23
SAP130	7.77	0.09
NUBP1	5.87	0.16
AMMECR1L	7.10	0.15
ZNF346	5.79	0.16
TUBB	8.86	0.23
FCF1	9.41	0.23
ZKSCAN5	4.94	0.22
TMUB2	8.13	0.24
ZNF143	8.26	0.28
TRIM39	6.40	0.30

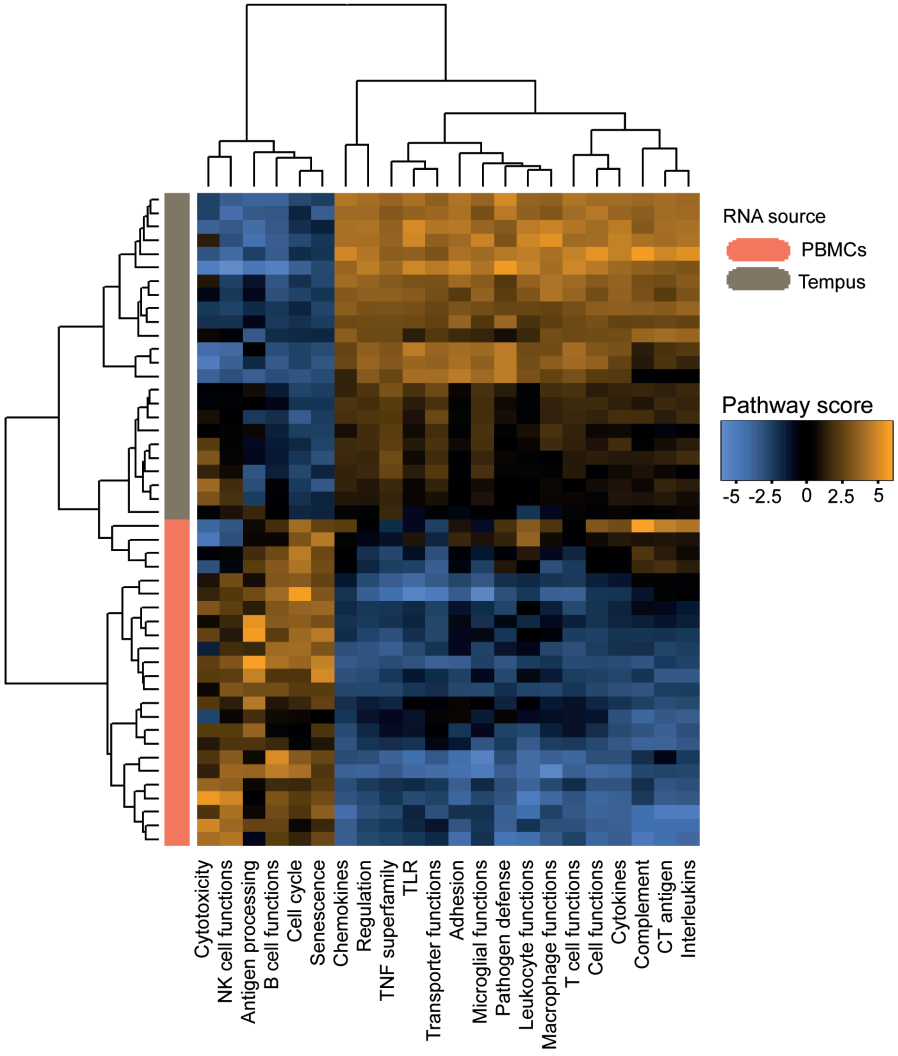
Supplementary Table 2. Genes used to define cell types.

Cell type	Definition genes	*P*
NK CD56dim cells	*IL21R, KIR3DL2, KIR3DL1* (discarded), *KIR_Inhibiting_ Subgroup_2* (discarded)	0.63
Cytotoxic cells	*GZMB, PRF1, KLRK1, GZMH, KLRB1, KLRD1, GNLY, GZMA, CTSW*	0
Dendritic cells	*CD209, HSD11B1, CCL13*	0.04
Regulatory T cells	*FOXP3*	NA
CD45+ cells	*PTPRC*	NA
Macrophages	*CD163, CD68, CD84* (discarded)	0.40
T cells	*CD3G, SH2D1A, CD6, CD3D, CD3E*	0
Mast cells	*TPSAB1, MS4A2*	0.29
Neutrophils	*CSF3R, S100A12*	0.02
Natural killer cells	*XCL2, NCR1*	0.50
Exhausted CD8+ cells	*CD244, EOMES, LAG3* (discarded)	0.60
B cells	*BLK, CD19, MS4A1, TNFRSF17* (discarded)	0.02
Th1 cells	*TBX21*	NA
CD8+ T cells	*CD8A, CD8B*	0.01

The *P*-value is calculated as the proportion (out of 1000 random gene sets) of gene sets that shows a better correlation than the gene sets defined by NanoString. NA = not applicable.



Supplementary Figure 1. Amount of detected genes, and overexpressed pathways and cell types for all samples individually. In most samples, Tempus provides more detected genes and more genes that are exclusively detected compared to PBMCs. In all samples, more pathways are overexpressed in Tempus compared to PBMCs. Only in 8/24 samples more cell scores are overexpressed in Tempus compared to PBMCs.



Supplementary Figure 2. Heatmap of pathway scores. There is perfect clustering of PBMC and Tempus samples based on relative pathway scoring.

Chapter 10

Serum cytokine levels predict tumor progression during FOLFIRINOX chemotherapy and overall survival in pancreatic cancer patients: a prospective multicenter study

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ABSTRACT

Background Biomarkers predicting treatment response may be used to stratify patients with pancreatic ductal adenocarcinoma (PDAC) for available therapies. The aim of this study was to evaluate the predictive value of circulating cytokines for FOLFIRINOX response and their association with overall survival (OS).

Methods Serum samples were collected before start and after the first cycle of FOLFIRINOX from patients with PDAC (*n*=83) of all disease stages. Overall, 34 circulating cytokines were analyzed with a multiplex immunoassay. In addition, changes in peripheral blood immune cell counts were determined by flow cytometry to correlate with differences in cytokine levels. Chemotherapy response was determined by CT scans with the RECIST 1.1 criteria, as disease control (*n*=64) or progressive disease (*n*=19) within eight cycles of FOLFIRINOX.

Results Patients with high serum IL-1RA concentrations after one cycle of chemotherapy were less likely to have tumor progression during FOLFIRINOX (OR 0.25, *P*=0.040). Increase of circulating IL-1RA concentrations correlated with increase of total, classical, and non-classical monocytes, and dendritic cells. After adjustment for chemotherapy response outcome and baseline CA19-9 level, serum concentrations of IL-7 (HR 2.14, *P*=0.010), IL-18 (HR 2.00, *P*=0.020), and MIP-1β (HR 0.51, *P*=0.025) after one cycle of FOLFIRINOX were correlated with OS.

Conclusions Circulating IL-1RA, IL-7, IL-18, and MIP-1β concentrations are predictive and prognostic biomarkers for FOLFIRINOX response in PDAC patients, suggesting an important role for specific immune cells in chemotherapy response and PDAC progression. Cytokine-based treatment might improve patient outcome and should be evaluated in future studies.

INTRODUCTION

FOLFIRINOX is a combined chemotherapy regime, including fluorouracil, leucovorin, irinotecan and oxaliplatin. It is currently the standard first-line treatment for locally advanced (LAPC) and metastatic pancreatic ductal adenocarcinoma (PDAC). Although the survival of patients with PDAC has improved with the implementation of this chemotherapy combination, the overall prognosis remains poor. Patients with LAPC have a median overall survival (OS) of approximately two years,[1] whereas metastatic disease patients a median OS of 11 months after FOLFIRINOX treatment.[2] Meanwhile, 60-70% of these patients will experience FOLFIRINOX-induced toxicity,[1-3] affecting their quality of life. Therefore, biomarkers with the ability to predict treatment response are urgently needed to personalize treatment and to avoid unnecessary toxicity.[4] Patients not responding to FOLFIRINOX might benefit from other types of chemotherapy, such as gemcitabine with nab-paclitaxel.

Cancer cells display pro-inflammatory properties, inducing a tumor-promoting environment.[5] Although PDAC is thought to be a 'cold' tumor, indicating the lack of active immune cells infiltrating the tumor,[6] systemic immune responses are present. Several systemic inflammation markers, such as the systemic immune-inflammation index,[7] neutrophil-to-lymphocyte ratio,[8] or Glasgow prognostic score[9] are of prognostic significance and alterations can be detected even prior to PDAC diagnosis.[10]

The stromal microenvironment of PDAC is a complex structure of extracellular matrix, fibroblasts, and inflammatory cells.[11] These inflammatory cells produce a variety of growth factors, cytokines, and chemokines.[11, 12] Cytokines are signaling molecules that play an important role in the interaction and function of cells. Cytokines are mainly produced by immune cells, but also normal epithelial cells, stromal cells, fibroblasts, and cancer cells can produce both pro-inflammatory and anti-inflammatory cytokines.[8] High levels of circulating immunosuppressive, tumor-promoting cytokines, such as transforming growth factor-beta (TGF-β), interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α), and lower levels of tumor-suppressive cytokines, e.g. IL-11, IL-12, and interferon-gamma (IFN-γ), have been found to correlate with poor prognosis in PDAC patients.[13-18] Circulating cytokine concentrations might reflect tumor aggressiveness and immune status associated with tumor progression.[19] Whether circulating cytokine levels can also predict the response to FOLFIRINOX is yet unknown. We hypothesized that dysregulation of the immune system, demonstrated by an increase of pro-inflammatory and decrease of anti-inflammatory cytokines, is prone to FOLFIRINOX non-response.

In this study, we evaluated the levels of circulating cytokine concentrations before and after one cycle of FOLFIRINOX, and assessed differences between patients with and without progressive disease at chemotherapy response evalution CT scans. In addition, peripheral blood immune cell subsets were measured and correlations with changes in cytokine concentrations were determined in order to affirm the origin of these cytokines. Also, the predictive value for early tumor progression during FOLFIRINOX and prognostic value for OS of individual cytokine markers was assessed.

MATERIALS AND METHODS

This article was written according to the Reporting recommendations for tumor marker prognostic studies (REMARK) guidelines.[20]

Study design

Patients were selected from the local pancreatic biobank at the Erasmus MC, Rotterdam (MEC-2015-085) and participated in two multicenter, prospective trials conducted in the Netherlands. Patients with resectable or borderline resectable PDAC participated in the randomized clinical trial PREOPANC-2 (Dutch trial register NL7094, MEC-2018-004) comparing neoadjuvant FOLFIRINOX chemotherapy to neoadjuvant gemcitabine-based chemoradiotherapy, followed by surgical resection of the primary tumor.[21] Patients with LAPC or metastatic PDAC participated in the iKnowIT study (Dutch trial register NL7522, MEC-2018-087), a prospective cohort study investigating the predictive value of circulating biomarkers. All trials were approved by the ethics committees of all participating centers: Erasmus MC, University Medical Center (Rotterdam, the Netherlands), Amsterdam UMC (Amsterdam, the Netherlands), Maasstad Hospital (Rotterdam, the Netherlands), and Medisch Spectrum Twente (Enschede, the Netherlands), and conducted in accordance with the declaration of Helsinki.

Patient selection

All patients had histologically confirmed PDAC and were treated with FOLFIRINOX between February 2015 and October 2019. Patients with resectable, borderline resectable, or locally advanced disease were scheduled for eight cycles of FOLFIRINOX and patients with metastatic disease for maximum twelve cycles of FOLFIRINOX, according to the PREOPANC-2 study protocol (resectable or borderline resectable disease) or the current standard of care in the Netherlands (LAPC and metastatic disease). Exclusion criteria were: age <18 years, co-medication with other chemotherapeutics, and previous treatment with FOLFIRINOX chemotherapy. A CT scan was performed before start of treatment and after each fourth cycle of chemotherapy to evaluate treatment response,

based on the Response Evaluation Criteria in Solid Tumours (RECIST) 1.1 criteria, as part of standard clinical practice. Differences in circulating cytokine levels were tested between patients with disease control patients, including those with stable disease, partial response or complete response to FOLFIRINOX, and progressive disease patients, if CT evaluation showed progression within eight cycles of FOLFIRINOX.

Patient characteristics, such as age, sex, stage of disease, laboratory results, CT scan evaluations, and follow-up data were retrieved from medical records by a medical doctor. Follow-up ended upon the death of the patient. Due to the explorative character of this study, no formal sample size calculation was performed.

Sample collection

Peripheral venous blood samples were collected before the start of chemotherapy and before the start of the second cycle of FOLFIRINOX, approximately two weeks later. Blood was collected in 10 mL serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and 10 mL EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Serum tubes were centrifuged at room temperature for 10 minutes at 1000g. Serum was then divided in aliquots and stored at -80 °C until further use. Freshly obtained whole blood from EDTA tubes was used within 24 hours to enumerate immune cell populations.

Cytokine detection

All serum samples were first analyzed with the ProcartaPlex Cytokine & Chemokine Convenience 34-Plex Human Panel 1A immunoassay (Invitrogen, Carlsbad, CA, USA, for detail see Supplementary Table 1), and measured using the Luminex MAGPIX system (Luminex, Austin TX, USA). Only cytokines detected in at least 70% of samples were used for further analyses. The cytokines IL-1β, IL-1RA, IL-2, and IL-18 were also measured using (high sensitivity) immunoassays from a different supplier (R&D systems, Minneapolis, MN, USA, Supplementary Table 1). In addition, soluble IL-2 receptor (sIL-2R) was quantified with enzyme-linked immunosorbent assay (ELISA; Diaclone, Besançon, France) as a sensitive marker for T-lymphocyte activation and regulator of IL-2-dependent cell function.[22, 23]

Serum samples were subjected to a maximum of three freeze-thaw cycles and thawed on ice prior to use. Supernatants were loaded on Luminex or ELISA plates at the recommended dilutions with standard protein controls, according to the manufacturer's instructions. For Luminex assays, cytokines were quantified by the analysis of raw data using xPONENT 4.2 software (Luminex, Austin, TX, USA). For the ELISA assay, standard curves were constructed and used to quantify sIL-2R. Further technical details are shown in Supplementary Table 1.

Immune cell enumeration

Flow cytometry was performed to quantify main granulocyte, monocyte, and lymphocyte subsets (Supplementary Table 2). Peripheral blood samples for immunophenotyping were available for 50/83 patients (60.2%). Whole blood was stained with monoclonal antibodies (MoAb) and after lysis of red blood cells analyzed by multi-color FCM on a BD 3-laser Celesta flow cytometer using FACSDiva 8.x software (Becton Dickinson, Franklin Lakes, NJ, USA). Absolute cell counts were determined using Flow-Count Fluorospheres (Beckman Coulter, Brea, CA, USA). The MoAb panel has been optimized, and compensated using Fluorescence minus one (FMO) controls.[24] Data were gated and analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

Statistical analysis

Detection rates of cytokines were compared using Fisher's exact tests. Absolute circulating cytokine concentrations and immune cell counts, and the percentual increase of cytokine concentrations and immune cell numbers were compared with Mann-Whitney U tests and Wilcoxon Signed Rank tests in case of paired data (before and after one cycle of FOLFIRINOX). Correlations between alterations in cytokine concentrations and immune cell numbers were determined with Pearson's correlation coefficient.

Univariable and multivariable binary logistic regression was performed to analyze the predictive value of circulating cytokine levels for tumor progression during FOLFIRINOX chemotherapy, adjusted for patient characteristics with known association with the outcome: stage of disease and baseline CA19-9 levels. Cytokine levels were dichotomized based on the median concentration for each individual cytokine per time point of measurement.

Overall survival (OS) was calculated as the time between the start of FOLFIRINOX and death. The prognostic value of circulating cytokine levels was tested with univariable and multivariable Cox regression analysis, including known prognostic factors: age, stage of disease, chemotherapy response, and baseline CA19-9 levels.

Only two-sided tests were used and *P*-values <0.05 were considered statistically significant. Data were analyzed using SPSS Statistics for Windows (version 25.0; IBM, Armonk, NY, USA).

RESULTS

Patient characteristics

In total, cytokine data obtained from 166 samples from 83 PDAC patients were available for analysis. Patient characteristics are presented in Table 1. The cohort consisted of patients from all disease stages: 33.7% was diagnosed with resectable/borderline resectable disease, 42.2% with LAPC, and 24.1% with metastatic disease. In this cohort, 19 (22.9%) patients showed progressive disease during FOLFIRINOX.

Table 1. Patient characteristics.

	All patients, *n*=83 (%)
Age (years), mean (range)	63 (41-80)
Sex, male	47 (56.6)
Stage of disease	
Resectable or borderline resectable	28 (33.7)
Locally advanced	35 (42.2)
Metastatic	20 (24.1)
Response* to FOLFIRINOX	
Stable disease	54 (65.1)
Partial response	10 (12.1)
Progressive disease	19 (22.9)
Response* to FOLFIRINOX, dichotomized	
Disease control	64 (77.1)
Progressive disease	19 (22.9)
Time point of CT evaluation progressive disease* (*n*=19)	
After cycle 1	1 (5.3)
After cycle 2	3 (15.8)
After cycle 3	2 (10.5)
After cycle 4	9 (47.4)
After cycle 6	1 (5.3)
After cycle 8	3 (15.8)
Number of cycles of FOLFIRINOX received, mean (range)	7 (1-12)
Baseline CA19-9 (kU/L), median (IQR)	320 (60-1296)

CA19-9 = carbohydrate antigen 19-9, IQR = interquartile range. *According to the RECIST 1.1 criteria.

Cytokine detection rates and treatment response outcome

Supplementary Table 3 gives an overview of the 34 cytokines and chemokines measured using a multiplex panel, showing their detection rate, mean concentrations with standard deviation, and median concentrations with interquartile range. GM-CSF, GRO-α,

IL-5, IL-12p70, IL-23, IL-31, and TNF-β were not detected in any of the samples. Eotaxin, IP-10, MIP-1β, RANTES, SDF-1α were detected in all serum samples. Comparisons of cytokine detection rates between patients with disease control and patients with progressive disease during FOLFIRINOX are presented in Table 2. Before the start of FOLFIRINOX, IL-1β (28.2% vs 5.3%) and IL-2 (18.8% vs 0%) were more often detected in samples from disease control patients then in samples from progressive disease patients (both *P*=0.059). After one cycle of FOLFIRINOX, IL-1RA was more often detected in patients with disease control (48.4%) compared to patients with progressive disease (21.1%, *P*=0.038).

To improve the detection rates of IL-1β, IL-1RA, IL-2, and IL-18, these cytokines were re-analyzed with high sensitivity singleplex or duplex immunoassay from a different manufacturer (Supplementary Table 1). Eotaxin, IL-7, IP-10, MCP-1, MIP-1β, RANTES, and SDF-1α all met the inclusion criteria of detection in >70% of samples and were selected for further analyses. Together with the sIL-2R, a total of eleven cytokines were used to create predictive and prognostic models.

Cytokine concentrations and treatment response outcome

IL-1β and IL-2 results were only available for 88 samples (*n*=44 patients). The other samples could not be used due to absence of detectable bead signals. With individual assays, IL-1β, IL-1RA, IL-18 and sIL-2R were detected in all available samples. IL-2 was detected in only 28.4% of samples and results of this cytokine will therefore not be further discussed.

There were no significant differences in median concentrations of any of the eleven cytokines (eotaxin, IL-1β, IL-1RA, sIL-2R, IL-7, IL-18, IP-10, MCP-1, MIP-1β, RANTES, SDF-1α) between disease control and progressive disease patients in samples drawn before the start or after one cycle of FOLFIRINOX (Figure 1A). Pro-inflammatory/tumor-promoting cytokines (eotaxin, IL-1β, IL-18) were found in higher concentrations in progressive disease patients, while disease control patients showed higher levels of anti-inflammatory/tumor-suppressive cytokines (IL-1RA, sIL-2R, IL-7).

In patients with disease control, IL-18 showed a larger increase during FOLFIRINOX (median increase 88.64%; IQR 46.05-141.86%) compared to patients with progressive disease (median increase 44.40%; IQR 10.60-81.29%), *P*=0.007), visualized in Figure 1B.

Predictive value of cytokine concentrations

To investigate the value of individual cytokine concentrations to predict disease progression during FOLFIRINOX, a binary logistic regression model was created. From

Table 2. Comparison of detection rates of cytokines and chemokines between patients with disease control and patients with progressive disease during FOLFIRINOX, measured with a multiplex Luminex panel. *P*-values are calculated by Fisher's exact tests.

Before start of FOLFIRINOX			
Cytokine / chemokine	**Detection rate disease control (%), *n*=64**	**Detection rate progressive disease (%), *n*=19**	***P***
IFN-γ	4 (6.3)	1 (5.3)	1.000
IL-1α	12 (18.8)	4 (21.1)	1.000
IL-1β	18 (28.1)	1 (5.3)	0.059
IL-1RA	2 (3.1)	1 (5.3)	0.547
IL-2	12 (18.8)	0 (0)	0.059
IL-6	1 (1.6)	1 (5.3)	0.408
IL-7	45 (70.3)	14 (73.7)	1.000
IL-10	2 (3.1)	0 (0)	1.000
IL-15	6 (9.4)	0 (0)	0.328
IL-17A	13 (20.3)	1 (5.3)	0.172
IL-18	29 (45.3)	6 (31.6)	0.428
IL-21	6 (9.4)	1 (5.3)	1.000
IL-22	5 (7.8)	0 (0)	0.584
IL-27	5 (7.8)	0 (0)	0.584
MCP-1	62 (96.9)	18 (94.7)	0.547
MIP-1α	18 (28.1)	5 (26.3)	1.000
TNF-α	10 (15.6)	0 (0)	0.107
After 1 cycle of FOLFIRINOX			
Cytokine / chemokine	**Detection rate disease control (%), *n*=64**	**Detection rate progressive disease (%), *n*=19**	***P***
IFN-γ	6 (9.4)	1 (5.3)	1.000
IL-1α	11 (17.2)	3 (15.8)	1.000
IL-1β	14 (21.1)	4 (21.1)	1.000
IL-1RA	31 (48.4)	4 (21.1)	0.038
IL-2	10 (15.6)	0 (0)	0.107
IL-6	4 (6.3)	2 (10.5)	0.616
IL-7	45 (70.3)	13 (68.4)	1.000
IL-10	3 (4.7)	0 (0)	1.000
IL-15	4 (6.3)	0 (0)	0.569
IL-17A	14 (21.9)	2 (10.5)	0.340
IL-18	44 (68.8)	11 (57.9)	0.416
IL-21	4 (6.3)	2 (10.5)	0.616
IL-22	4 (6.3)	1 (5.3)	1.000
IL-27	5 (7.8)	0 (0)	0.584
MCP-1	62 (96.9)	17 (89.5)	0.223
MIP-1α	13 (20.3)	4 (21.1)	1.000
TNF-α	8 (12.5)	1 (5.3)	0.677

IFN = interferon, IL = interleukin, IL-1RA = interleukin-1 receptor antagonist, MCP = monocyte chemoattractant protein, MIP = macrophage inflammatory protein, TNF = tumor necrosis factor.

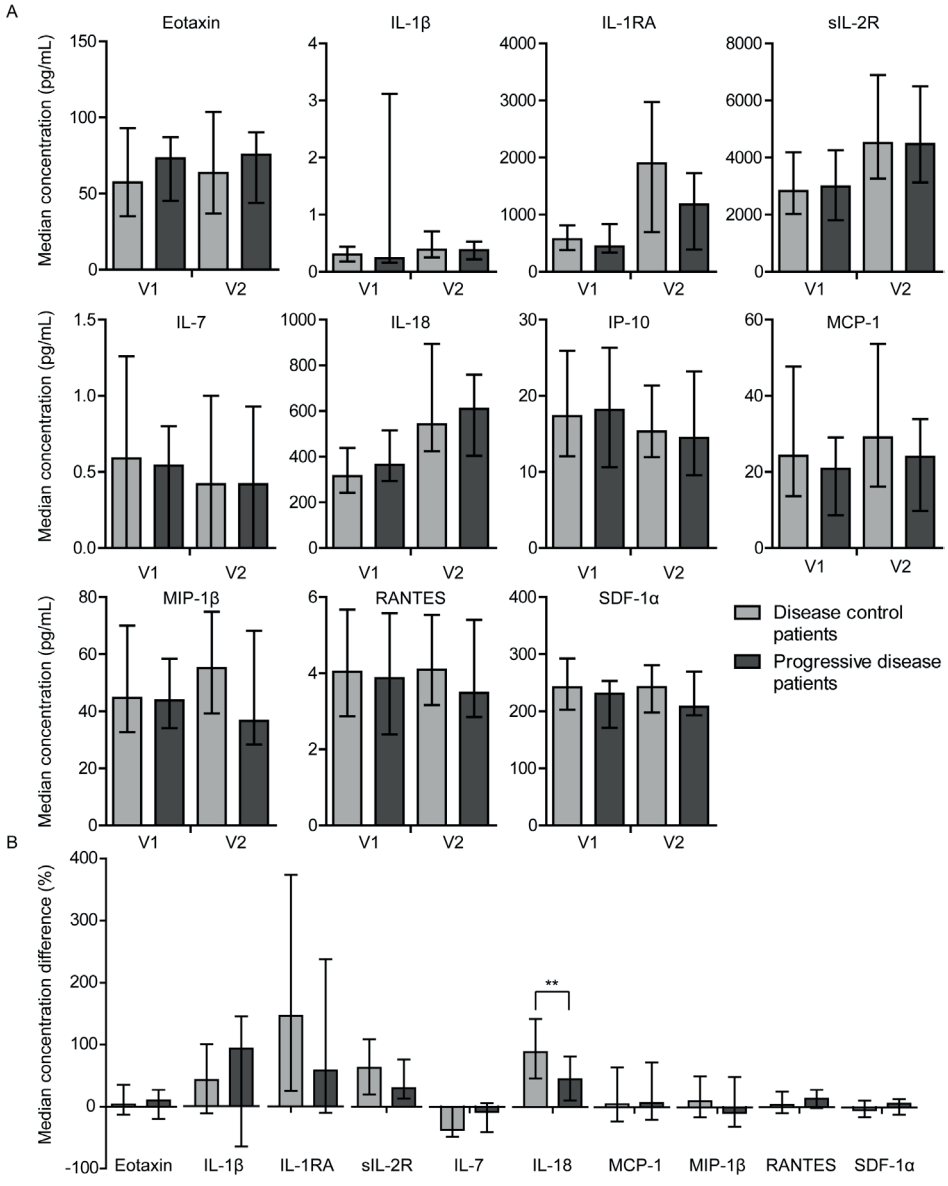

Figure 1. Differences in circulating cytokine concentrations between patients with disease control and patients with progressive disease during FOLFIRINOX treatment.

A. Concentrations of serum cytokines in patients with disease control (n=64) and patients with progressive disease (n=19) before start of FOLFIRINOX (V1) and after one cycle of FOLFIRINOX (V2).

B. Percentage increase of serum cytokine concentrations after one cycle of FOLFIRINOX. Treatment-induced IL-18 increase was higher in patients with disease control compared to patients with progressive disease after FOLFIRINOX. ** = P<0.01, calculated with Mann-Whitney U test.

univariable analyses, the variables IL-1RA concentration after one cycle of FOLFIRINOX (OR 0.19, 95% CI 0.06-0.63), IL-18 before the start of FOLFIRINOX (OR 2.62, 95% CI 0.88-7.74), an increase of IL-18 (OR 0.33, 95% CI 0.11-0.94), and an increase of MIP-1β (OR 0.37, 95% CI 0.13-1.08) were selected for multivariable analyses. In this patient cohort, stage of disease and baseline CA19-9 level did not predict early tumor progression. The results of the univariable and multivariable analyses are presented in Table 3. In multivariable analysis, only IL-1RA concentration after one cycle of FOLFIRINOX remained an independent predictor of FOLFIRINOX response (OR 0.25; 95% CI 0.07-0.094, *P*=0.040). Patients with IL-1RA concentrations above the median of the measurements showed a lower risk of early tumor progression during FOLFIRINOX.

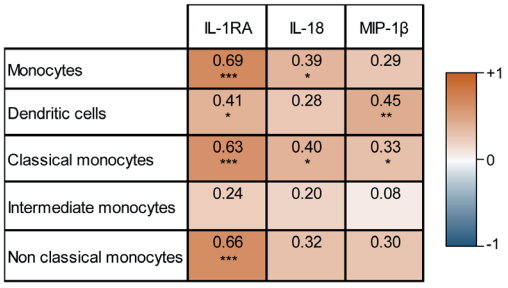

Figure 2. Correlation matrix of monocyte-related serum cytokine concentration increase and circulating monocyte cell number increase after one cycle of FOLFIRINOX.
Increasing IL-1RA, IL-18, and MIP-1β concentrations after one cycle of FOLFIRINOX showed significant correlations with the increase of several subsets of monocytes. * = *P*<0.05, ** = *P*<0.01, *** = *P*<0.001, calculated with Pearson's correlation.

Table 3. Univariable and multivariable binary logistic regression model for the prediction of early tumor progression during FOLFIRINOX.

	Univariable		**Multivariable**	
Variable	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
IL-1RA after 1 cycle of FOLFIRINOX				
<median	Ref		Ref	
>median	0.19 (0.06-0.63)	0.007	0.25 (0.07-0.94)	0.040
IL-18 before start of FOLFIRINOX				
<median	Ref		Ref	
>median	2.62 (0.88-7.74)	0.083	2.08 (0.63-6.92)	0.231
IL-18 increase during 1 cycle of FOLFIRINOX				
≤50%	Ref		Ref	
>50%	0.33 (0.11-0.94)	0.038	0.66 (0.119-2.27)	0.506
MIP-1β increase during 1 cycle of FOLFIRINOX				
No	Ref		Ref	
Yes	0.37 (0.13-1.08)	0.069	0.73 (0.21-2.48)	0.729

CI = confidence interval, IL = interleukin, IL-1RA = interleukin-1 receptor antagonist, MIP = macrophage inflammatory protein, OR = odds ratio, Ref = reference.

Correlation between cytokines and immune cells

IL-1RA, IL-18, and MIP-1β, predictors of early tumor progression in univariable analysis, are cytokines produced by monocytes. Therefore a correlation matrix was made between the percentual increase of serum concentrations of these cytokines and the percentual increase of monocyte cell numbers, based on results available from 50 patients (Figure 2). The increase of circulating IL-1RA concentrations correlated significantly with an increase of total monocytes (Pearson's r=0.69, *P*<0.001), classical monocytes (r=0.63, *P*<0.001), non-classical monocytes, and dendritic cells (r=0.41, *P*=0.011) in the peripheral blood (r=0.66, *P*<0.001). IL-18 correlated with total monocytes (r=0.39, *P*=0.015), and classical monocytes (r=0.40, *P*=0.014). MIP-1β correlated with dendritic cells (r=0.45, *P*=0.004), and classical monocytes (r=0.33, *P*=0.046).

Overall, an increase in all determined immune cell types was observed after one cycle of FOLFIRINOX. There were no statistically significant differences in cell numbers before the start of FOLFIRINOX, after one cycle of FOLFIRINOX or in increase over time between disease control and progressive patients (Figure 3). However, we could detect a trend towards a stronger increase of immune-promoting cells (e.g. neutrophils, B cells, NK cells, and monocytes) in disease control patients, together with a slight increase of immunosuppressive cells (e.g. γδ T cells) in progressive disease patients. However, there was also an increasing trend in T cells visible, including tumor-suppressive T helper cells (CD4+) and cytotoxic T cells (CD8+), in progressive disease patients.

Cytokines associated with overall survival

The median follow-up time was 16.5 months for patients alive at last follow-up. Median OS for the total cohort was 12.5 months. In univariable analyses, IL-1RA concentration after FOLFIRINOX (HR 0.64 for concentrations above the median of measurements), sIL-2R before the start of FOLFIRINOX (HR 1.55, >median), IL-7 after FOLFIRINOX (HR 1.57, >median), IL-18 before (HR 1.56, >median) and after FOLFIRINOX (HR 1.57, >median), and MIP-1β after FOLFIRINOX (HR 0.63, >median) were statistically significant predictors for OS, as shown in Table 4. In multivariable analysis and after adjustment for baseline CA19-9 and RECIST treatment response outcome, IL-7 (HR 2.14; 95% CI 1.20-3.80, *P*=0.010), IL-18 (HR 2.00; 95% CI 1.11-3.60, *P*=0.020), and MIP-1β (HR 0.51; 95% CI 0.28-0.92, *P*=0.025) concentrations measured after one cycle of FOLFIRINOX remained significant prognostic factors for OS after FOLFIRINOX treatment. Patients with a high level of IL-7 and IL-18, and low level of MIP-1β were at risk for shorter OS. Stage of disease was not a prognostic variable in this patient cohort.

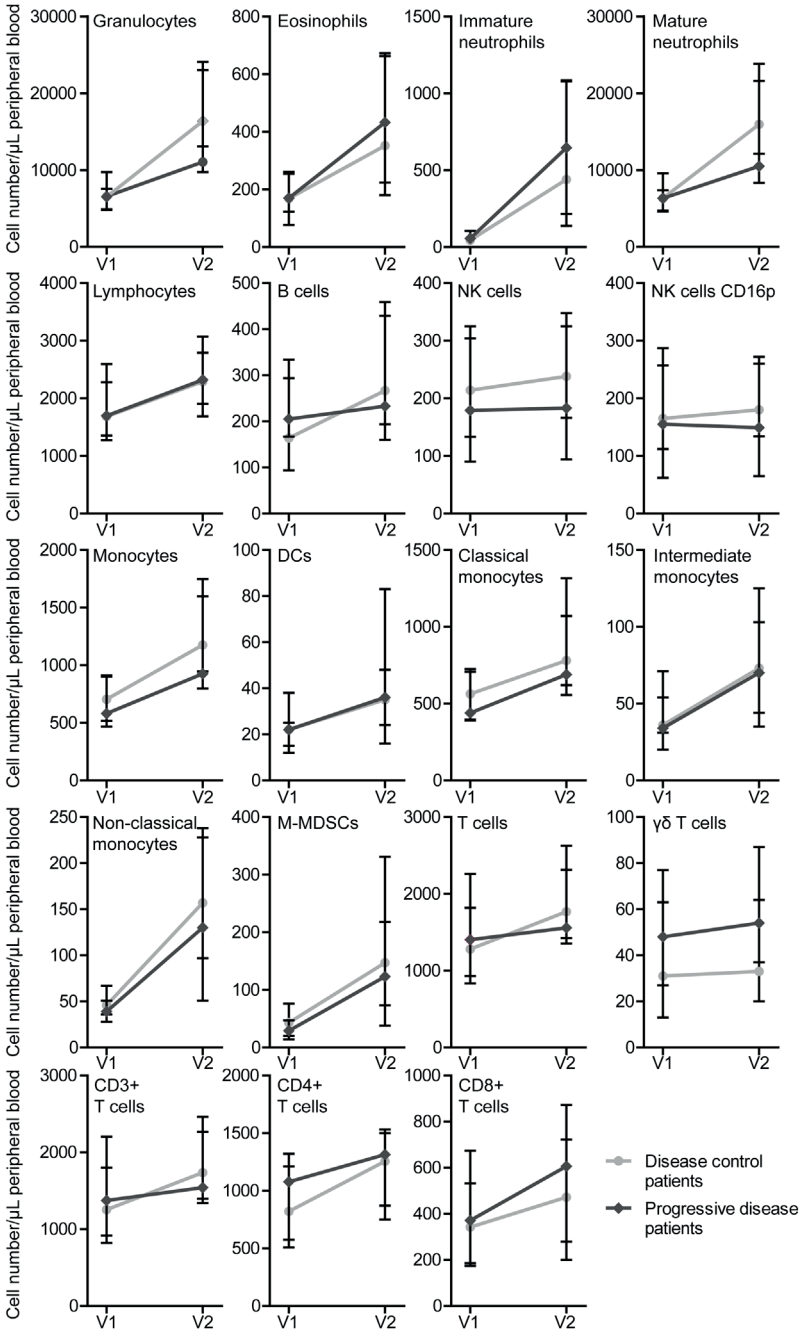

Figure 3. Differences in circulating immune cell numbers in peripheral blood between patients with disease control and patients with progressive disease during FOLFIRINOX treatment.

Circulating immune cell numbers in patients with disease control and patients with progressive disease before start of FOLFIRINOX (V1) and after one cycle of FOLFIRINOX (V2). Cell numbers of all investigated circulating immune cells showed an increase during one cycle of FOLFIRINOX. Data is presented as median cell numbers with interquartile ranges.

Table 4. Univariable and multivariable Cox proportional hazards model for overall survival (OS) after FOLFIRINOX.

	Univariable		Multivariable	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
CA19-9 at baseline (per 100 kU/L)	1.00 (1.00-1.01)	0.021	1.00 (1.00-1.01)	0.044
RECIST response outcome				
Disease control	Ref		Ref	
Progressive disease	7.37 (3.84-14.15)	<0.001	6.66 (2.96-14.98)	<0.001
IL-1RA after 1 cycle of FOLFIRINOX				
<median	Ref		Ref	
>median	0.64 (0.38-1.09)	0.098	0.93 (0.47-1.85)	0.834
sIL-2R before start of FOLFIRINOX				
<median	Ref		Ref	
>median	1.55 (0.93-2.59)	0.093	1.07 (0.59-1.93)	0.823
IL-7 after 1 cycle of FOLFIRINOX				
<median	Ref		Ref	
>median	1.57 (0.93-2.65)	0.090	2.14 (1.20-3.80)	0.010
IL-18 before start of FOLFIRINOX				
<median	Ref		Ref	
>median	1.56 (0.93-2.60)	0.090	1.12 (0.62-2.02)	0.714
IL-18 after 1 cycle of FOLFIRINOX				
<median	Ref		Ref	
>median	1.57 (0.94-2.62)	0.084	2.00 (1.11-3.60)	0.020
MIP-1β after 1 cycle of FOLFIRINOX				
<median	Ref		Ref	
>median	0.63 (0.38-1.05)	0.076	0.51 (0.28-0.92)	0.025

CA19-9 = carbohydrate antigen 19-9, CI = confidence interval, HR = hazard ratio, IL = interleukin, IL-1RA = interleukin-1 receptor antagonist, MIP = macrophage inflammatory protein, Ref = reference, sIL2R = soluble interleukin-2 receptor.

DISCUSSION

In this prospective multicenter study, we investigated serum cytokine concentrations in PDAC patients treated with FOLFIRINOX and the correlations of these cytokines with treatment response and prognosis. We found that most cytokines showed increasing concentrations after one cycle of FOLFIRINOX compared to baseline samples. However, IL-18 showed a stronger increase in patients responding to treatment. Also, IL-1RA serum concentrations after one cycle of FOLFIRINOX predicted treatment response, with higher concentrations associated with a lower risk of early tumor progression during FOLFIRINOX. In addition, IL-18, IL-7, and MIP-1β after one cycle were prognostic factors for OS.

Our results support the hypothesis that prognosis in cancer patients, including patients with PDAC, is at least partially determined by the activation level of the immune system and its response to cancer cells.[5] We found a clear serum cytokine pattern in patients with disease control and patients with progressive disease during FOLFIRINOX. Favorable, anti-inflammatory cytokines, such as IL-1RA, IL-7, and MIP-1β were detected in higher concentrations in responding patients, while pro-inflammatory, tumor-promoting cytokines, such as IL-18 and IL-1β, were higher in patients with early progressive disease, though not statistically significant.

The correlation we observed between IL-1RA, IL-18 and MIP-1β with different monocyte subsets might indicate that differences in cytokine concentrations are related to differences in the immune environment and cellular response to chemotherapy. Though, the strong increase of T cells, including tumor-suppressive T helper cells (CD4+) and cytotoxic T cells (CD8+), in progressive disease patients, was unexpected. However, it is not clear whether the increase of all the investigated cell types might be explained by the increased cell proliferation in reaction to chemotherapy-induced cell death, by the administration of granulocyte colony-stimulating factor (G-CSF) after every chemotherapy cycle, or as a result of the proliferative effect on immune cells of FOLFIRINOX. Furthermore, we did not investigate the functional status and exhaustion rate of CD4+ and CD8+ cell populations, which is crucial in further studies.

The results found in this study are affirmed by existing literature on circulating cytokine levels and outcome in PDAC patients. Circulating IL-18 levels were previously found to increase during treatment with gemcitabine in combination with 5-FU or oxaliplatin, and higher IL-18 levels were associated with shorter OS.[25] IL-1β is thought to facilitate tumor growth, angiogenesis, and metastasis in PDAC and may therefore negatively influence patient prognosis.[13, 15] Both IL-18 and IL-1β are associated with objective gemcitabine-based chemotherapy response.[13, 26] Also, MIP-1β and RANTES have been shown to associate with PDAC patient outcome.[27, 28]

An interesting finding is that most cytokines with predictive or prognostic value in our study relate to the nuclear factor-κB (NF-κB) pathway, which plays a crucial role in the regulation of a plethora of inflammatory genes.[29]. The NF-κB signaling cascade is usually activated by pathogens, damaged tissue, and necrotic cells, and it includes a negative feedback loop, regulating its own activity.[30] In cancer environments the NF-κB signaling pathway is continuously activated, resulting in constant production of large amounts of pro-inflammatory cytokines, including IL-1, IL-2, IL-18, and VEGF. Activation of the NF-κB pathway promotes all hallmarks of cancer: tumor cell proliferation and survival, angiogenesis, metastasis, and immune suppression.[29, 31] NF-κB might also participate

in macrophage polarization, converting tumor-suppressing M1 macrophages into tumor-promoting M2 macrophages.[31] TNF-α and IL-1β, of which the latter was found in higher concentrations in our patients with poor prognosis, are known activators of the canonical NF-κB pathway.[32] Also, several DNA mutations can activate the pathway,[32, 33] including *KRAS* and *TP53* mutations which are found in almost all PDAC tumors.[34] IL-1RA, the natural antagonist of IL-1, may regulate activation of the NF-κB pathway, thus reducing the negative effects of the pathway and improving patient prognosis.[33]

However, other cytokines which are also controlled by NF-κB were not detected at all or only detected in a limited number of patient samples. For example, TNF-α, IL-6, and IL-8, cytokines that have been shown to be upregulated in PDAC patients,[13-15, 35] were only detected in a limited number of samples. The relatively low serum cytokine concentrations and therefore lack of detection, is probably the most important limitation of this study. Although PDAC might not be as immunogenic as other cancers such as lung cancer,[36] which might explain the relative low cytokine levels, the cytokine concentrations measured in our study also seem to be low in comparison to other studies with PDAC patients.[13, 15, 27] This may be related to the immunoassay used to detect the cytokines. In general, highly sensitive methods using relatively large amounts of input material usually show higher concentrations compared to multiplex methods using only small volumes. We chose to start our pilot project with a broad multiplex panel to screen for 34 different cytokines. With this approach, only small serum volumes were needed, which was an advantage because of the limited availability of serum from our patients, included in large multicenter, prospective studies.

In this pilot study, the sample size was relatively small and cytokine concentrations varied widely between patients. Most of our findings did therefore not reach statistical significance and we could only highlight some trends in cytokine and cell count differences between treatment response groups. Subgroup analyses for the individual disease stages could not be performed due to the limited number of patients. Nevertheless, our data support that future clinical trials should aim at collection of sufficient amounts of serum or plasma which could be used for biomarker analyses, including circulating cytokines.

The future clinical implications of this study should include the evaluation of the additive effects of cytokine-based therapy on chemotherapy response and survival in PDAC patients. Studies with mouse models have already shown promising results of treatment with anakinra, an FDA-approved human IL-1RA protein drug used for rheumatoid arthritis. Anakinra inhibits IL-1 and by that the NF-κB pathway, reducing proliferation, migration, and invasion of PDAC cells, and IL-1 neutralization sensitizes cancer cells for

immunotherapy and chemotherapy.[33, 37] At this moment, two clinical trials are investigating the benefit of anakinra in addition to FOLFIRINOX (ClinicalTrials.gov identifier: NCT02021422) or gemcitabine chemotherapy (NCT02550327) in PDAC patients. Restoring the imbalance of tumor-promoting and tumor-suppressing components of the immune system might be the future of PDAC treatment.

CONCLUSION

Circulating IL-1RA cytokine concentrations predict FOLFIRINOX response and IL-18, IL-7, and MIP-1β are prognostic factors for OS in PDAC patients. This indicates that activation and changes in the systemic immune response play an important role in chemotherapy response and PDAC progression. Cytokine-based treatment might improve patient outcome and should be evaluated in future studies.

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SUPPLEMENTARY FILES

Supplementary Table 1. Technical details of the used immunoassays.

Cytokine/chemokine	Immunoassay	Company	Amount of serum used	Dilution
Eotaxin, GM-CSF, GRO-α, IFN-α, IFN-γ, IL-1α, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, SDF-1α, TNF-α, TNF-β	Luminex ProcartaPlex Cytokine & Chemokine Convenience 34-Plex Human Panel 1A	Invitrogen	12.5 µL	1:2
IL-1β, IL-2	Luminex Performance Human High Sensitivity Cytokine Magnetic Panel B	R&D systems	25 µL	1:2
IL-1RA	Luminex Performance Human XL Cytokine Discovery Magnetic Panel	R&D systems	17.5 µL	1:2
IL-18	Luminex Human Magnetic Assay	R&D systems	25 µL	1:2
sIL-2R	ELISA	Diaclone	20 µL	1:5

GM-CSF = granulocyte-macrophage colony-stimulating factor, GRO = growth-regulated oncogene, IFN = interferon, IL = interleukin, IL-1RA = interleukin-1 receptor antagonist, IP = interferon γ-induced protein, MCP = monocyte chemoattractant protein, MIP = macrophage inflammatory protein, RANTES = regulated upon activation, normal T cell expressed and secreted, SDF = stromal cell-derived factor, sIL2R = soluble interleukin-2 receptor, TNF = tumor necrosis factor.

Supplementary Table 2. Flow cytometry markers used for immunophenotyping of whole blood.

	Markers
Granulocytes	
Eosinophils	$CD45^+$ SSC $CD15^+$ $CD16^-$
Mature neutrophils	$CD45^+$ SSC $CD15^{high}$ $CD16^{high}$
Immature neutrophils	$CD45^+$ SSC $CD15^+$ $CD16^+$
Monocytes	
Classical monocytes	$CD45^+$ SSC $CD14^+$ $CD16^-$
Intermediate monocytes	$CD45^+$ SSC $CD14^+$ $CD16^+$
Non-classical monocytes	$CD45^+$ SSC $CD14^-$ $CD16^+$
Dendritic cells	$CD45^+$ SSC $CD14^-$ $CD16^-$ $CD11c^+$
Myeloid-derived suppressor cells (MDSCs)	CD45+ SSC CD14+ CD16- CD11b+ HLA-DR low
Lymphocytes	
B cells	$CD45^+$ SSC $CD3^-$ $CD19^+$
NK cells	$CD45^+$ SSC $CD3^-$ $CD56^+$ $CD16^{+/-}$
T cells	$CD45^+$ SSC $CD3^+$
αβ T cells	$CD45^+$ SSC $CD3^+$ $TCR\alpha\beta^+$ $CD4^+/CD8^+$
γδ T cells	$CD45^+$ SSC $CD3^+$ $TCR\gamma\delta^+$

CD = cluster of differentiation, NK = natural killer, SSC = side scatter.

Supplementary Table 3. Overview of serum cytokine detection rates and concentrations of the 34 cytokines and chemokines measured with the ProcartaPlex multiplex immunoassay.

	Before start of FOLFIRINOX (*n*=83)			After 1 cycle of FOLFIRINOX (*n*=83)		
Cytokine/ chemokine	**Detection rate (%)**	**Mean concentration, pg/mL (SD)**	**Median concentration, pg/ mL (IQR)**	**Detection rate (%)**	**Mean concentration, pg/mL (SD)**	**Median concentration, pg/ mL (IQR)**
Eotaxin	83 (100)	67.28 (36.24)	61.11 (36.06-90.96)	83 (100)	71.92 (40.93)	72.12 (37.23-94.49)
GM-CSF	0 (0)	NA	NA	0 (0)	NA	NA
GRO-α	0 (0)	NA	NA	0 (0)	NA	0.00 (0.00-0.00)
IFN-α	2 (2.4)	0.09 (0.72)	0.00 (0.00-0.00)	0 (0)	NA	NA
IFN-γ	5 (6.0)	0.15 (0.70)	0.00 (0.00-0.00)	7 (8,4)	0.14 (0.55)	0.00 (0.00-0.00)
IL-1α	16 (19.3)	9.14 (48.28)	0.00 (0.00-0.00)	14 (16,9)	8.98 (48.82)	0.00 (0.00-0.00)
IL-1β	19 (22,9)	0.82 (3.98)	0.00 (0.00-0.00)	18 (21,7)	0.57 (1.99)	0.00 (0.00-0.00)
IL-1RA	3 (3,6)	12.79 (82.01)	0.00 (0.00-0.00)	35 (42.2)	623.14 (1279.31)	0.00 (0.00-1063.19)
IL-2	12 (14,5)	1.22 (5.50)	0.00 (0.00-0.00)	10 (12.0)	0.47 (1.47)	0.00 (0.00-0.00)
IL-4	1 (1.2)	NA	NA	0 (0)	NA	NA
IL-5	0 (0)	NA	NA	0 (0)	NA	NA
IL-6	2 (2,4)	0.03 (0.19)	0.00 (0.00-0.00)	6 (7,2)	0.25 (1.29)	0.00 (0.00-0.00)
IL-7	59 (71.1)	0.85 (1.09)	0.54 (0.00-1.11)	58 (69.9)	0.71 (1.07)	0.42 (0.00-0.93)
IL-8	0 (0)	NA	NA	2 (2.4)	0.78 (4.32)	0.00 (0.00-0.00)
IL-9	1 (1.2)	NA	NA	1 (1.2)	NA	NA
IL-10	2 (2,4)	0.12 (1.10)	0.00 (0.00-0.00)	3 (3,6)	0.14 (1.23)	0.00 (0.00-0.00)
IL-12p70	0 (0)	NA	NA	0 (0)	NA	NA
IL-13	1 (1.2)	NA	NA	3 (3.6)	0.06 (0.39)	0.00 (0.00-0.00)
IL-15	6 (7,2)	3.74 (15.56)	0.00 (0.00-0.00)	4 (4.8)	1.29 (6.41)	0.00 (0.00-0.00)
IL-17A	14 (16,9)	2.13 (7.77)	0.00 (0.00-0.00)	16 (19.3)	1.20 (6.54)	0.00 (0.00-0.00)
IL-18	35 (42.2)	2.64 (5.02)	0.00 (0.00-2.95)	55 (66.3)	9.88 (12.84)	4.56 (0.00-14.72)

Supplementary Table 3. Overview of serum cytokine detection rates and concentrations of the 34 cytokines and chemokines measured with the ProcartaPlex multiplex immunoassay. (continued)

	Before start of FOLFIRINOX (*n*=83)			After 1 cycle of FOLFIRINOX (*n*=83)		
Cytokine/ chemokine	**Detection rate (%)**	**Mean concentration, pg/mL (SD)**	**Median concentration, pg/ mL (IQR)**	**Detection rate (%)**	**Mean concentration, pg/mL (SD)**	**Median concentration, pg/ mL (IQR)**
IL-21	7 (8.4)	25.35 (132.29)	0.00 (0.00-0.00)	6 (7.2)	20.53 (108.79)	0.00 (0.00-0.00)
IL-22	5 (6.0)	63.68 (361.96)	0.00 (0.00-0.00)	5 (6.0)	36.21 (211.76)	0.00 (0.00-0.00)
IL-23	0 (0)	NA	NA	0 (0)	NA	NA
IL-27	5 (6.0)	2.99 (15.35)	0.00 (0.00-0.00)	5 (6.0)	2.45 (16.38)	0.00 (0.00-0.00)
IL-31	0 (0)	NA	NA	0 (0)	NA	NA
IP-10	83 (100)	19.94 (13.03)	17.43 (11.66-26.31)	83 (100)	18.42 (10.27)	15.23 (11.87-21.71)
MCP-1	80 (96.4)	29.77 (23.70)	23.86 (11.47-44.51)	79 (95.2)	34.26 (27.53)	27.02 (15.48-50.01)
MIP-1α	23 (27.7)	3.44 (8.74)	0.00 (0.00-1.34)	17 (20.5)	2.84 (8.50)	0.00 (0.00-0.00)
MIP-1β	83 (100)	67.04 (102.81)	42.17 (33-37-65.20)	83 (100)	72.88 (93.55)	50.82 (34.94-72.11)
RANTES	83 (100)	4.39 (2.06)	4.03 (2.75-5.64)	83 (100)	4.60 (2.00)	4.08 (3.13-5.42)
SDF-1α	83 (100)	299.25 (345.30)	239.78 (200.65-285.37)	83 (100)	282.83 (297.43)	232.54 (197.30-277.98)
TNF-α	10 (12.0)	0.28 (1.33)	0.00 (0.00-0.00)	9 (10,8)	0.19 (0.97)	0.00 (0.00-0.00)
TNF-β	0 (0)	NA	NA	0 (0)	NA	NA

GM-CSF = granulocyte-macrophage colony-stimulating factor, GRO = growth-regulated oncogene, IFN = interferon, IL = interleukin, IL-1RA = interleukin-1 receptor antagonist, IP = interferon γ-induced protein, IQR = interquartile range, MCP = monocyte chemoattractant protein, MIP = macrophage inflammatory protein, NA = not applicable, RANTES = regulated upon activation, normal T cell expressed and secreted, SD = standard deviation, SDF = stromal cell-derived factor, TNF = tumor necrosis factor.

Chapter 11

General summary,
discussion,
and future perspectives

GENERAL SUMMARY

Personalized medicine is a treatment modality in which the choice for treatment, drug type and dosage, and other medical interventions are selected based on the specific needs of an individual patient. Treatment response, and especially drug response, shows large inter-patient variability.[1] The response to drugs relies on three components: the drug responsiveness of tissues, the drug concentration at the site of interest, and disease- or tumor-specific characteristics.[2] Drug response is for the most part determined by genetics of the patient, and the genetics of the tumor in patients with malignant disease, which is called pharmacogenomics.[1] Traditional chemotherapy schedules include non-specific cytotoxic drugs, damaging both cancer cells as well as normal cells.[3] The response to treatment and chemotherapy-induced toxicity is quite unpredictable. However, with the discovery of drug response biomarkers, including genetic, epigenetic, and immunologic markers, a more targeted approach of cancer treatment might become possible.[3]

For patients with advanced pancreatic ductal adenocarcinoma (PDAC), FOLFIRINOX chemotherapy is the current standard-of-care.[4, 5] However, not all patients benefit from this treatment; some might show early tumor progression already during treatment[4-6] while experiencing severe FOLFIRINOX-induced side effects.[5-7] Patient stratification for chemotherapeutic treatment of patients with PDAC is unfortunately not yet possible. Therefore, the aim of this thesis was to investigate genetic and immunologic biomarkers that can predict early tumor progression during FOLFIRINOX to identify these patients before the start or in an early phase of chemotherapy.

We first describe in this thesis the clinical outcome of patients treated with FOLFIRINOX (**Part I**). Studies investigating the (survival) benefit in patients treated with new chemotherapy regimens, including studies on FOLFIRINOX, are usually performed in selected patient cohorts. The benefit of treatment might be overestimated due to exclusion of patients with poor prognosis. Next to that, large patient cohorts are not necessarily comparable to patients in daily practice in our own hospital. In **Chapter 2**, therefore, we investigated the response rates and survival benefit of FOLFIRINOX in unselected, real-world patient cohorts in a multicenter, retrospective study. We found that the response rate was rather low (14-18%) in patients with PDAC treated with FOLFIRINOX, comparable to the existing literature.[4, 5, 8] However, in most patients the disease stabilized with this treatment. Especially in patients with locally advanced pancreatic cancer (LAPC), the outcome was acceptable; more than 77% of patients showed disease control during and immediately after FOLFIRINOX. In **Chapter 3**, the quality of life of LAPC patients after FOLFIRINOX was evaluated. Because of the toxicity-profile of FOLFIRINOX,[6, 7] physicians

and patients are often anxious and holding back to start treatment, but its long-term impact is not clear. In the cohort that we evaluated, quality of life was excellent after FOLFIRINOX. LAPC patients reported high global health scores, high scores for functioning scales, such as cognitive and emotional functioning, and almost no disease-related symptoms (e.g. pain, diarrhea, appetite loss). Overall FOLFIRINOX outcome was better than we expected.

In **Part II** of this thesis, we first gave an overview of currently known predictive biomarkers for chemotherapy response in PDAC patients (**Chapter 4**). Although several promising candidate markers have been evaluated for FOLFIRINOX and gemcitabine response prediction, no circulating biomarkers have been properly validated or are currently being used in clinical practice. This shows again the need for large, prospective biomarker studies. In **Chapter 5**, we described the results of next-generation sequencing of circulating DNA in patients with PDAC. With this technique, genetic mutations in DNA originating from tumor cells (ctDNA) can be detected in blood plasma. Patients with larger and more aggressive tumors, with more tumor-burden, have a larger amount of ctDNA in their circulation.[9, 10] We hypothesized that the amount of ctDNA might also be higher in patients that do not respond to FOLFIRINOX treatment. Next to that, specific ctDNA mutations might be associated with poor outcome, comparable to DNA mutations detected in tissue.[11-14] The results of our study show that the combination of a *TP53* ctDNA mutation with a homozygous *TP53* Pro72Arg germline variant is a predictive marker for early tumor progression during FOLFIRINOX and this combination is associated with poor OS. In the total cohort of 48 patients, five had this circulating combination marker and all five of these patients showed early disease progression during FOLFIRINOX.

We continued our search for predictive biomarkers for FOLFIRINOX response in **Chapter 6** with circulating microRNAs (miRNAs), important regulators of gene expression and cell pathways.[15-17] We found that patients with progressive disease showed higher expression of serum miR-373-3p in samples before start of FOLFIRINOX, and lower expression of serum miR-194-5p in samples after one cycle of FOLFIRINOX, compared to patients with disease control. These miRNAs were not associated with overall survival. Because a large proportion of miRNAs originate from (circulating) immune cells,[18, 19] we further investigated the role of miRNAs in **Chapter 7**. In this chapter we aimed to identify miRNAs associated with systemic inflammation markers, such as the neutrophil-to-lymphocyte ratio (NLR) and systemic immune-inflammation index (SII). Systemic inflammation markers are poor prognostic factors in PDAC patients,[20, 21] however the underlying mechanism is still unclear. miRNAs might illuminate how alterations in the immune system influence patient outcome. In patients with resectable PDAC, circulating miR-338-3p

and miR-199b-5p concentrations were correlated to the absolute neutrophil count in the peripheral blood. An association with survival could not be confirmed in this small cohort, but future research including miRNAs could elucidate new therapeutic targets, for example by targeting immune alterations, to improve patient outcome.

Part III of this thesis began with an overview of circulating immunologic biomarkers associated with PDAC prognosis (**Chapter 8**). In patients with PDAC, multiple components of the immune system in the peripheral blood have been found to be of prognostic significance. However, the function of immune cells, cytokines, and other immune components is often ambiguous and can act either as tumor-suppressors as well as tumor-promotors, depending on many environmental factors. For this reason, research involving immune markers is difficult, but the immune system is probably one of the key players in cancer progression and treatment response and therefore it is important to investigate immunologic biomarkers in further studies to identify new therapeutic targets for immune therapy. Immune-related gene expression is one of multiple methods to monitor changes in the immune system. However, measuring gene expression profiles in peripheral blood samples is complicated because of the relative instability and low quality of circulating RNA. In **Chapter 9**, we aimed to determine the best source of RNA for immune-related gene expression research: RNA from stabilized whole blood or RNA isolated from peripheral blood mononuclear cells (PBMCs), which is a pure pellet of isolated immune cells. Apparently, despite dilution of the blood by the stabilizing reagent, stabilized whole blood enables more comprehensive measurement of gene expression profiles compared to PBMC RNA. An RNA stabilizing reagent preserves gene activity and enables detection of a higher number of immune-related genes. It also ensures that RNA from all sources is preserved, including RNA from all blood components and secreted RNA that is washed away when purifying PBMCs. If both sample types are available, we would recommend using stabilized whole blood for immune gene expression analyses in future studies.

Next to gene expression from immune-related genes, immunologic research should also include other immune biomarkers. The number of immune cells is of course very important, because it tells us something about the level of activation of the immune system. However, immune cells interact with each other and influence each other's function by their signaling molecules: cytokines.[22, 23] Cytokines are mainly produced by immune cells, but also normal epithelial cells, stromal cells, fibroblasts, and cancer cells can produce both pro-inflammatory and anti-inflammatory cytokines.[24] Cytokines can polarize immune cells and might act as tumor-promoting factors or tumor-suppressive factors under different circumstances. Prognosis of patients with PDAC has previously been linked to cytokine levels.[25-29] Next to prognostic value of circulating cytokines,

we showed in **Chapter 10** that circulating cytokine levels can also predict treatment outcome. High circulating interleukin-1 receptor antagonist (IL-1RA) is associated with lower risk of early tumor progression during FOLFIRINOX. This cytokine is an important regulator of the NF-κB pathway. In cancer environments, the NF-κB signaling pathway is continuously activated, resulting in constant production of large amounts of pro-inflammatory cytokines, including IL-1, IL-2, IL-18, and VEGF. IL-1RA is the natural antagonist of IL-1 and reduces the cancer-promoting effects of IL-1 and other negative effects of the NF-κB pathway on the hallmarks of cancer.[30, 31] The effect of IL-1RA treatment should be studied in future clinical trials.

DISCUSSION AND FUTURE PERSPECTIVES

The research in this thesis shows that there are many promising biomarkers in PDAC management to be further explored. Biomarkers are not only useful tools to select patients for available treatment options, but prognostic and predictive biomarkers could also be an important starting point for the development of new therapies. However, despite the increasing research interest and rapid evolvement in this area, PDAC biomarkers are still far from clinical implementation.

Combination of biomarkers

The development and progression of cancer, including PDAC, is always the result of a combination of genetic changes and the loss of normal cellular regulatory processes, including immunologic responses.[32]

Cancer usually originates from one or multiple genetic mutations, leading to uncontrolled cell growth. Though, the fact that different genetic mutations can lead to the same type of cancer and, at the same time, tumors with similar genetic alterations develop and progress completely different, strongly suggests that other genetic, epigenetic, and immunologic factors play an important role too. It is plausible that different genetic mutations all need different escape mechanisms to successfully induce malignant transformation. Therefore, it is unlikely that one perfect biomarker can predict treatment response or prognosis in patients with PDAC. Instead, it is very important to investigate the value of the combination of biomarkers. The results of this thesis illuminate some biomarker combinations that should be further investigated and validated. Interestingly, we found multiple biomarkers involved in the same pathways and cell processes.

We showed with our ctDNA analyses that the combination of a *TP53* ctDNA mutation and a homozygous *TP53* germline variant is a better predictor of early tumor progression

during FOLFIRINOX and a better prognostic marker for overall survival than a *TP53* tumor mutation on its own. The hypothesis is that a slightly different folding of the P53 protein, induced by this common *TP53* germline variant, increases the aggressiveness and metastatic potential of *TP53* mutated tumors. This common single nucleotide polymorphism (SNP) does not seem harmless anymore. Maybe more SNPs can be identified to be involved in PDAC progression; SNPs we thought to be 'benign'. For the interpretation of clinical trials it is very important to know that genotypes might influence the response to treatment. However, genotypes and SNP frequencies might be ethnicity-specific and results from biomarker studies across the world might not directly be applicable to your own patient cohort. It would be interesting to combine mutational analyses from tissue and peripheral blood with SNP analyses in future biomarker studies.

Malignant properties of genetic mutations might also depend on changes in miRNA expression. Because miRNAs are important regulators of gene expression,[15, 16] they might also influence expression of mutation-induced oncogenic genes, such as *KRAS* and *TP53*. Earlier studies have shown that miR-373, in our cohort associated with early tumor progression during FOLFIRINOX, can bypass the wild-type *TP53* tumor suppressing response in patients with oncogenic RAS-induced tumorigenesis.[33, 34] Instead of analyzing treatment response, progression, or survival in patient subgroups based on disease stage, it would perhaps make more sense to group patients based on the mutational status of their tumors. For most of the patients included in our biomarker cohorts, tumor mutational status was unknown, because tumor mutation sequencing is not part of the diagnostic routine. For future PDAC biomarker studies, it is crucial to analyze and combine multiple candidate-biomarkers, including at least tumor mutational status.

Study design

Since we believe that treatment response and disease development and progression depend on multiple (patient-specific) factors, including tumor environment factors, it is necessary that research is performed on patient samples. Although these samples are sometimes hard to obtain and show a large variability in clinical and biological characteristics, it is the only way to investigate the connection and interaction of different genetic or immunologic components. Research involving cell lines does not allow for that, since there is no tumor microenvironment.[35] Also mouse models are not usable to look for new biomarkers to be used in the treatment of PDAC patients. Malignant tumors from mouse models are affected by tumor microenvironment, but this is different from the human situation.[36]

The ideal study design for biomarker research would be to incorporate this as a side study in clinical trials. Including blood draws or tissue sampling as part of the study

protocol provides valuable patient samples that can be used to monitor treatment effect and for identification and validation of biomarkers. Next to that, with obtaining samples from patients included in randomized clinical trials, a control cohort will be available for biomarker analyses. To make sure that a biomarker is really predictive for response to treatment, you should prove that this effect is treatment-specific. Otherwise, this biomarker acts only as a prognostic tool.[37] Unfortunately, a control cohort was not available when conducting the research described in this thesis. For that reason, we could only investigate biomarkers for their value to predict early tumor progression during treatment. We were not able to tell whether patients with early tumor progression had aggressive tumors, resulting in early progression, or whether these tumors are resistant to FOLFIRINOX specifically. When validating our results in a new, larger patient population, we would include a control cohort of patients treated with a different chemotherapy regimen, such as gemcitabine with nab-paclitaxel.

Collaborations

For successful research on biomarkers and the validation and clinical implementation of those, a large number of patient samples in combination with a complete set of clinical data of the included patients is required. To make this work, it is essential to collaborate with other research groups. In the Netherlands, we have a national collaboration program, the Dutch Pancreatic Cancer Group (DPCG), to establish and coordinate PDAC treatment standards and guidelines, new clinical trials, and clinical data registry. The DPCG would be the ideal platform to standardize patient sample collection in addition to clinical trials. Though, the exchange of previously collected patient material would of course also be of great value and could help increase sample sizes in new and ongoing biomarker studies.

Next to collaboration between research groups, collaboration between clinicians and researchers might be of paramount importance. Too often, communication between these two is lacking; clinicians identify urgent clinical research questions, but do not have the biomedical expertise to execute laboratory experiments, while basic researchers are unaware of the needs in the clinic. Better coordination between the clinic and the lab would improve the quality of the research and fasten the implementation of found results.

CONCLUSION

In summary, predictive biomarkers are urgently needed to stratify patients for available treatments and to identify new targets for personalized medicine. Although we

discovered multiple promising candidate-biomarkers for FOLFIRINOX response in PDAC patients, the results need to be thoroughly validated in larger, independent patient cohorts, including control patients, to establish chemotherapy-specific treatment response. Future research should focus on biomarker combinations, rather than exploration of individual genetic, epigenetic, or immunologic features since they all cohere. The collaboration between clinicians and basic researchers is of great importance to establish new biomarker studies.

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Chapter 12

Nederlandse samenvatting

ACHTERGROND

Met "personalized medicine", of gepersonaliseerde geneeskunde, wordt getracht een behandelschema op te stellen dat specifiek gericht is op een individuele patiënt. Immers reageert niet elke patiënt op dezelfde manier op behandeling, met name als het gaat om geneesmiddelen. Hoe een patiënt precies reageert op medicatie is van verschillende factoren afhankelijk. Onder andere (epi)genetische factoren van de patiënt zelf en specifieke genetische mutaties van de tumor die wordt behandeld, hebben invloed op het resultaat van behandeling. Biomarkers zijn meetbare parameters in het bloed of tumorweefsel die informatie kunnen geven hoe een ziekte, bijvoorbeeld alvleesklierkanker, zich ontwikkelt of reageert op de behandeling. Deze biomarkers kunnen ons in de toekomst helpen om behandelstrategieën te personaliseren.

Patiënten met alvleesklierkanker worden vaak pas gediagnosticeerd als de ziekte in een vergevorderd stadium is. De primaire tumor is dan te ver doorgegroeid in met name omliggende bloedvaten, of is al naar andere organen uitgezaaid. In deze gevallen worden patiënten meestal behandeld met chemotherapie om hun leven te verlengen, terwijl dit niet tot genezing leidt. Op dit moment is FOLFIRINOX de standaardbehandeling bij gevorderde alvleesklierkanker, omdat het de meeste overlevingswinst geeft. Echter, niet alle patiënten reageren op deze behandeling; de tumor blijft soms ongecontroleerd doorgroeien. Tegelijkertijd ondervinden veel mensen ernstige bijwerkingen van FOLFIRINOX, wat nadelige effecten heeft op de kwaliteit van leven in hun laatste levensfase.

Het doel van het onderzoek in dit proefschrift is gericht op het vinden van biomarkers in het bloed die kunnen voorspellen welke patiënten reageren op FOLFIRINOX-behandeling. Met een dergelijke biomarker kan voorkomen worden dat mensen die geen baat zullen gaan hebben bij chemotherapie wel ernstige bijwerkingen hiervan krijgen. Tevens maakt een biomarker het mogelijk om patiënten te identificeren die beter een andere behandeling kunnen ondergaan.

SAMENVATTING

Dit proefschrift is opgebouwd uit drie delen. In **deel I** hebben we eerst onderzocht hoe groot daadwerkelijk het probleem is met betrekking tot de respons op FOLFIRINOX-behandeling. In **hoofdstuk 2** hebben we laten zien dat weliswaar bij weinig patiënten de tumor kleiner wordt door FOLFIRINOX chemotherapie, maar dat bij de meeste patiënten wel stabiele ziekte wordt bereikt en de tumor niet verder groeit. Bij een agressieve tumor zoals alvleesklierkanker is dat hoopgevend en zorgt het waarschijnlijk voor verlenging

van de levensduur. Daarnaast is het heel belangrijk om deze data te gebruiken bij het optimaal informeren van patiënten. Verwachte uitkomsten van FOLFIRINOX zijn vooralsnog bijna altijd gebaseerd op resultaten van klinische studies waarbij alleen patiënten met een relatief goede gezondheid mee mogen doen.

In **hoofdstuk 3** hebben we de kwaliteit van leven beschreven van patiënten met alvleesklierkanker na FOLFIRINOX-behandeling. Aangezien FOLFIRINOX veel bijwerkingen kan hebben, zijn behandelend artsen en patiënten soms huiverig om te beginnen met behandeling uit angst dat dit mogelijk wel levensverlenging geeft, maar teveel impact op kwaliteit van leven heeft. Echter, patiënten met gevorderde alvleesklierkanker gaven aan dat zij hun kwaliteit van leven heel goed vinden na het afronden van FOLFIRINOX. Ze hadden weinig bijwerkingen van de chemotherapie en de ziekte zelf, waren goed in staat voor zichzelf te zorgen en hadden vrijwel geen slaapproblemen of pijn. Deze uitkomsten van kwaliteit van leven waren beter dan verwacht.

In **deel II** van dit proefschrift verdiepten we ons in genetische biomarkers die FOLFIRINOX-respons kunnen voorspellen. Alhoewel er reeds meerdere kandidaat-biomarkers zijn ontdekt, is er nog geen enkele ook direct bruikbaar in de praktijk (**hoofdstuk 4**). Om deze reden zijn er grote, prospectieve studies nodig om biomarkers te ontdekken én te valideren voor toekomstig gebruik.

Eén van de typen biomarkers die wij hebben onderzocht, is circulerend tumor DNA (ctDNA). Met deze techniek kunnen DNA-mutaties van de tumor in het bloed worden gedetecteerd. Uit eerder onderzoek is gebleken dat het kunnen detecteren van DNA-mutaties geassocieerd is met een slechte prognose. Grotere, agressievere tumoren geven meer DNA af aan de bloedbaan. Wij onderzochten daarom de hypothese dat patiënten die niet reageren op FOLFIRINOX, de non-responders, ook meer ctDNA in hun bloed hebben of specifieke ctDNA mutaties die een tumor ongevoelig maken voor chemotherapie in **hoofdstuk 5**. In ons patiëntencohort bleek dat patiënten met een combinatie van mutaties in het *TP53* gen nooit reageerden op FOLFIRINOX en waarbij de tumor dus heel snel progressief groeide. Mocht deze bevinding gevalideerd worden in een toekomstig, groter cohort van patiënten, dan kan deze marker gebruikt kunnen worden om non-responders voor start van FOLFIRINOX te identificeren. Op die manier kunnen zij een andere behandeling krijgen waar ze wellicht wel baat bij hebben.

Met hetzelfde doel hebben we ook microRNAs onderzocht (**hoofdstuk 6**). MicroRNAs zijn korte strengen RNA die genexpressie en cel processen reguleren. We hebben verschillen gevonden tussen patiënten die reageerden op FOLFIRINOX-therapie en patienten die al progressie vertoonden tijdens behandeling: progressieve patiënten hadden

relatieve overexpressie van circulerend miR-373-3p en onderexpressie van circulerend miR-194-5p. Ook deze factoren zouden dus in de toekomst gebruikt kunnen worden voor het selecteren van patiënten voor FOLFIRINOX.

Aangezien veel microRNAs in het bloed afkomstig zijn van immuuncellen, hebben we in **hoofdstuk 7** onderzocht op welke manier we microRNA-expressie kunnen koppelen aan systemische inflammatie in patiënten met pancreascarcinoom. Systemische inflammatie, zich uitend in verhoogde ontstekingswaarden en –cellen in het bloed, is geassocieerd met een slechte prognose. Wij hebben laten zien dat een verhoogd aantal neutrofielen gepaard gaat met een verhoogde expressie van circulerend miR-338-3p en miR-199b-5p. MicroRNAs lijken dus een rol te spelen in chronische ontsteking bij kanker en progressie van ziekte.

Deel III beschrijft circulerende immunologische biomarkers: markers afkomstig van het afweersysteem. Daarin (**hoofdstuk 8**) bespreken we eerst welke immunologische markers al bekend zijn in patiënten met alvleesklierkanker. Deze markers zijn weliswaar geassocieerd met de prognose van patiënten, maar er is nog weinig bekend over de predictieve waarde van dergelijke markers voor de respons op therapie. Bij onderzoek naar immunologische biomarkers kunnen verschillende aspecten worden onderzocht. Bijvoorbeeld immuuncellen, hun signaalmoleculen cytokines, of immuunsysteem-geassocieerde genexpressie kunnen allemaal invloed hebben op de respons op therapie.

Om onderzoek te kunnen doen naar genexpressie van immuuncellen of immuungeassocieerde genen is het belangrijk om te weten welk materiaal het beste resultaat geeft. Hiervoor hebben we in **hoofdstuk 9** een vergelijking gemaakt tussen RNA geïsoleerd uit enkel immuuncellen (PBMCs) en RNA geïsoleerd uit volbloed. PBMCs hebben als voordeel dat het RNA wat hieruit geïsoleerd wordt niet gemengd is met RNA van overige cellen, zoals rode bloedcellen. Echter, RNA is zeer instabiel en is mogelijk al in kwaliteit gedaald op het moment van analyse. Volbloed dat gestabiliseerd wordt met een RNA stabilisator bevat dan weliswaar meer dan alleen immuuncellen, maar de kwaliteit van het RNA blijft waarschijnlijk intact. Dit is precies wat onze resultaten lieten zien: als RNA uit gestabiliseerd volbloed wordt geanalyseerd, krijgt men veel betere én uitgebreidere resultaten vergeleken met RNA uit PBMCs. Wij bevelen daarom ook gestabiliseerd volbloed aan om te gebruiken in toekomstig onderzoek waarbij immunologische genexpressie wordt onderzocht.

Eén van de typen immunologische biomarkers die wij hebben onderzocht, is cytokines. Dit zijn signaalmoleculen die onder andere immuuncellen uitscheiden om met elkaar te communiceren en zo te activeren of te inhiberen. In **hoofdstuk 10** hebben we beschre-

ven hoe circulerende levels van cytokines ook therapie-uitkomsten kunnen voorspellen. Grote hoeveelheden van interleukine-1 receptor antagonist (IL-1RA) beschermen tegen vroege ziekteprogressie tijdens FOLFIRINOX chemotherapie. Deze cytokine reguleert het NF-κB proces. In patiënten met kanker is dit proces continu gestimuleerd waardoor er heel veel andere, inflammatoire cytokines worden geproduceerd. Dit leidt tot een staat van chronische inflammatie waarin kankercellen zich optimaal voelen en daardoor makkelijk groeien en uitzaaien. IL-1RA kan deels dit proces afremmen en daarmee ook de negatieve effecten ervan.

In conclusie hebben we dus meerdere veelbelovende biomarkers gevonden die mogelijk de respons op chemotherapie kunnen voorspellen. Om deze biomarkers ook in de praktijk te kunnen gebruiken is echter meer onderzoek nodig ter validatie van deze resultaten.

Appendices

List of publications
List of contributing authors
PhD portfolio
Acknowledgements
Curriculum vitae

LIST OF PUBLICATIONS

Serum miR-373-3p and miR-194-5p are associated with early tumor progression during FOLFIRINOX treatment in pancreatic cancer patients: a prospective multicenter study.
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PHD PORTFOLIO

Name PhD student	Fleur van der Sijde	**PhD period**	2017-2021
Erasmus MC department	Surgery	**Promotor**	Prof. dr. C.H.J. van Eijck
Research school	MolMed	**Supervisors**	Dr. E.E. Vietsch
			Dr. D.A.M. Mustafa

	Year	ECTS
Courses		
Basic Course OpenClinica	2017	0.5
Elektronische Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (eBROK)	2018	1.5
Research Integrity	2018	0.3
Basic Introduction Course on SPSS	2018	1.0
Vectra Course	2018	1.0
Biostatistical Methods I: Basic Principles, part A	2018	2.0
Basic Course on Flow Cytometry	2018	0.2
Biomedical Research Techniques	2018	1.5
Introduction in GraphPad Prism Version 6	2018	0.3
Basic Human Genetics course: Genetics for Dummies	2018	0.6
Survival Analysis Course	2018	0.6
UCSC Genome Browser	2019	0.3
Presentations		
MolMed day, Rotterdam – *poster presentation*	2019	0.3
EAHPBA, Amsterdam – *poster presentations*	2019	0.6
SEOHS, Amsterdam – *oral presentation*	2019	1.0
IHPBA, virtual conference – *poster presentation*	2020	0.3
NVGE DDD voorjaarsdag, virtual conference – *oral presentation*	2021	1.0
European Pancreatic Club, virtual conference – *oral presentation*	2021	1.0
EAHPBA, virtual conference – *oral presentation*	2021	1.0
Conferences attended		
International Hepato-Pancreato-Biliary Association Geneva, Switzerland	2017	1.0
Wetenschapsdag Heelkunde, Erasmus MC Rotterdam, the Netherlands	2018	1.0
Wetenschapsdag Heelkunde, Erasmus MC Rotterdam, the Netherlands	2019	1.0
MolMed day Rotterdam, the Netherlands	2019	1.0
South-West Pancreatic Cancer Care Rotterdam, the Netherlands	2019	1.0
European-African Hepato-Pancreato-Biliary Association Amsterdam, the Netherlands	2019	1.0
Stichting Experimenteel Onderzoek Heelkundige Specialismen Amsterdam, the Netherlands	2019	1.0
Wetenschapsdag Heelkunde, Erasmus MC Rotterdam, the Netherlands	2020	1.0
International Hepato-Pancreato-Biliary Association Virtual conference	2020	1.0
Nederlandse Vereniging voor Gastroenterologie voorjaarsdag Virtual conference	2021	1.0

European Pancreatic Club Virtual conference	2021	1.0
European-African Hepato-Pancreato-Biliary Association Virtual conference	2021	1.0
Teaching		
BLS examinations	2017-2018	0.5
Supervision master student (F. Grevers)	2018-2019	0.5
Supervision HLO students (R. Schraauwen, O. Anaz)	2019-2020	1.0
Other		
Surgical Journal Club of Clinical Oncology	2017-2021	1.0
Peer reviewing for medical journals	2020-2021	3.0

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CURRICULUM VITAE

Fleur van der Sijde was born in Dordrecht on September 14th 1992. She graduated from Johan de Witt gymnasium in Dordrecht in 2010 after which she started medical school at the Erasmus University Rotterdam, being admitted through decentralized selection. She combined her study with her work in a medical student team for both the Surgery and Gastroenterology department at the Erasmus Medical Center Rotterdam and was a waitress/barista during the weekends. After obtaining her medical degree in 2016, she started working as a resident not in training (ANIOS) at the department of Surgery at the Erasmus Medical Center. The academic atmosphere there strengthened her interest in research and she got the opportunity to start a PhD program under supervision of prof. dr. Van Eijck, which has led to this thesis. Because of her broad interest in very diverse medical specialties, she will become a general practitioner. However, she hopes to continue with (pancreatic cancer) research in the future.