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Prognostic value of circulating T-lymphocyte subsets in advanced pancreatic cancer patients treated with mFOLFIRINOX or gemcitabine

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ABSTRACT

Advanced pancreatic ductal adenocarcinoma (PDAC) is commonly treated with a chemotherapy combination of mFOLFIRINOX or gemcitabine. However, predictive and prognostic factors for choosing a more appropriate treatment strategy are still lacking. This study aimed to evaluate how chemotherapy changes immune system parameters and whether these changes influence survival outcomes. We sought to identify an easily accessible marker to help choose the appropriate treatment. Patients with PDAC who were suitable for systemic chemotherapy were eligible for the study. Peripheral blood samples were obtained at baseline and after two months of treatment. Lymphocyte subsets were measured using flow cytometry. Correlation with clinical features and survival analyses were performed. In total, 124 patients were enrolled in this study. Seventy patients were treated with mFOLFIRINOX and 50 with gemcitabine monotherapy. Four patients could not be treated because of rapid deterioration. During overall survival analysis (OS), significant factors included age, Eastern Cooperative Oncology Group (ECOG) performance status, differentiation grade G3, carcinoma antigen (CA) 19–9 more than 100 kU/L, absolute white blood cell count, CD3 + CD8+, and CD8 + CD57-T lymphocytes. Natural killer CD3-CD56 + CD16 + and CD3-CD56 + CD16- and T regulatory CD4 + FOXP3 + and CD3 + CD56 + cells differed during treatment, but these differences did not influence the survival results. At baseline, CD8 + CD57- T lymphocyte count demonstrated a clear independent impact on progression-free survival and OS. Gemcitabine showed better survival in patients with extremely low baseline CD8 + CD57- levels. Therefore, circulating CD3 + CD8 + and CD8 + CD57- cells measured before treatment in PDAC may be considered prognostic and predictive biomarkers.

1. Introduction

Pancreatic cancer is highly fatal, especially in the third and fourth stages, where the 5-year survival rate does not exceed 10 % [1]. The advanced-stage mFOLFIRINOX regimen was used after proving its advantages in clinical trials [2]. Treatment with mFOLFIRINOX was earlier compared with gemcitabine treatment. Gemcitabine is currently combined with nab-paclitaxel. Chemotherapy is known to have an immunomodulatory effect; however, the impact of chemotherapeutic agents may depend on the immune tumour microenvironment (TME). In pancreatic ductal adenocarcinoma (PDAC), chemotherapy can change the immune profile in the microenvironment by enhancing the cross-presentation of tumour-associated antigens, thereby decreasing Tregs [3] and strengthening NK cell cytotoxicity [4].

The function of pancreatic cancer and the immune system is not fully understood; immunotherapy is ineffective due to the immunosuppressive TME, but its effects in combination are being studied. Different immunotherapy strategies have been rejected, but other clinical trials, including combinations of conventional chemotherapy [5] and vaccines [6] are still ongoing. Currently, immunotherapy is known to be beneficial in MSI-H tumours, but it only accounts for < 1 % of cases [7]. Infiltration with CD3+, CD4+, CD8+, and PD1 + T cells and

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macrophages is considered prognostic, but MSI-H/dMMR remains the only predictive factor for immunotherapy [6].

For a long time, the serum marker carcinoma antigen (CA) 19–9 has been used in routine clinical practice; however, some parameters of the immune system should be monitored in combination with immunotherapy. In other cancerous diseases where immunotherapy has already proven to be efficient, the predictive significance of circulating lymphocytes becomes comprehensible [8–11].

Meta-analysis data confirmed that infiltration by CD3 + and CD8 + cells improves survival, and increased FoxP3 + leads to poorer prognosis [12]. Additionally, CD8 + cells are the best effectors of long-term memory [13]. Increased presence of CD8 + cells in PDAC is associated with prolonged survival [14]. Lower circulating CD8 + levels may be associated with more advanced disease [15].

Through the involvement of macrophages and NK cells, CD4 + promotes cellular immunity [13], and increased numbers in the TME are favourable prognostic factors [14]. Tregs are immunosuppressive components that inhibit cytotoxic responses in the TME [16] and are associated with a poor prognosis [17]. Increased counts of circulating regulatory T cells are predictors of worse outcomes [15]. Infiltration with NK cells is relatively low in the PDAC TME, and there is a significantly lower activity of circulating NK cells than in a healthy population [18]. They have a reduced quantity of cytotoxicity receptors [13], increased CD16 expression in the CD56 dim subset, and the CD56dimCD16-negative NK population is associated with less disease recurrence [19].

Several researchers have studied how chemotherapy alters immune cells in PDAC. FOLFIRINOX was studied for neoadjuvant use, and higher CD8 + expression was detected [20], exhaustion of regulatory T cells and myeloid-derived suppressor cells in the TME was observed, and intratumoural infiltration by CD4 + T cells [20,21] and NK cells had prognostic value [21]. Neoadjuvant FOLFIRINOX can decrease regulatory T cells and Th2, but increase Th1 and CD8 T cells in the peripheral blood [22]. In advanced PDAC, using the same chemotherapy regimen, 30 % of changes were exposed to regulatory T cells and PD-1 + T cells during the first month of chemotherapy [23].

CD8 + infiltration may be predictive of chemotherapy. High infiltration by CD8 + lymphocytes coincides with better disease-free survival and overall survival (OS) in gemcitabine-treated patients but not in the observation arm [24].

Based on these findings, we hypothesised that different chemotherapy regimens could affect the immune system differently, leading to differences in survival. Many recent studies have concentrated on TME research, and circulating lymphocytes that are achievable from the patient's blood should also be of interest.

2. Materials and methods

2.1. Enrolled participants

This prospective study was conducted at the Vilnius University Hospital Santaros Klinikos. Patients with PDAC who were suitable for systemic chemotherapy between February 2018 and April 2021 were eligible for the study. Before chemotherapy, all the patients provided informed consent for participation in the study. Approval was obtained from the Vilnius Regional Bioethics Committee. Patients were excluded if they were ineligible for chemotherapy due to concomitant diseases or poor ECOG status, received anti-cancer treatment for advanced or metastatic PDAC earlier, or had co-existing diseases of the immune system.

2.2. Treatment of patients

Patients with advanced and metastatic disease and a confirmed pathological diagnosis of PDAC were evaluated at diagnosis by a multidisciplinary team, including a surgeon, medical and radiotherapy oncologist, and radiologist. Owing to jaundice, some patients were treated with endoscopic retrograde cholangiopancreatography and biliary stent insertion. Some of the patients underwent palliative bypass surgery. Material for pathological evaluation was obtained during surgery or core needle biopsies from primary or metastatic sites. mFOLFIRINOX (fluorouracil + leucovorin + irinotecan + oxaliplatin and gemcitabine) regimens were used as first-line treatment, and the regimen was chosen according to a patients' ECOG status and concomitant diseases. The National Comprehensive Cancer Network recommends these two regimens for patients with excellent and poor performance statuses. The treating oncologist chose the appropriate chemotherapy regimen and dosage. Chemotherapy lasted a maximum of 12 cycles (14-days per cycle) for mFOLFIRINOX and six cycles for gemcitabine-based regimens (28-days per cycle).

2.3. Data collection

Before chemotherapy, demographics, comorbidities (particularly autoimmune diseases), ECOG status, body mass index (BMI), serum haemoglobin, platelet count, albumin, serum glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, and CA 19–9 levels were recorded in all the patients. First, data were collected every-two months until disease progression, and multiple visits were planned. Later, we considered studying materials from two visits only.

The status of the disease volume was evaluated using radiological imaging, including three-phase, high-resolution chest and abdomen, pelvic contrast-enhanced CT scan, and biochemical testing, including CA 19–9 was done. Progressive disease is characterised by radiologic progression or exacerbation of clinical symptoms, usually with increased CA 19–9 levels, based on institutional protocols.

Automated complete blood counts from patients' venous blood samples were performed before flow cytometric analysis. We used the Sysmex XN-1000 haematology analyser to detect the white blood cell (WBC) population counts.

Further, patient samples for the eight-colour cytometric analysis were prepared by the lysed whole blood technique using monoclonal antibodies for surface markers. The cellular membrane permeabilization step was added to the sample preparation for staining intracellular markers. To obtain absolute cell counts for sample staining and incubation, we used the Lyse No Wash technique with TruCount tubes (Becton Dickinson). Antibodies for detecting surface and intracellular antigens were added to 100 μL of peripheral ethylenediaminetetraacetic acid anticoagulated venous blood. We used the following monoclonal antibodies: CD45-V450, CD19-V500, CD3-PerCP, CD4-APC-H7, CD8-PE-Cy7, CD25-FITC, CD127-PE, cFoxP3-APC, CD16-PE, and CD56-APC. The CD4/CD8 ratio was calculated from laboratory data.

Stained samples were acquired on a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA), and data were analysed using FACS Diva version 8.0.2. software (Becton Dickinson). We used a sequential gating technique and biexponential dot plots to reveal the desired cell population hierarchies for data display.

2.4. Outcome measures

The primary objective of this study was to investigate how changes in lymphocyte subpopulation count during chemotherapy influence progression-free survival (PFS) and OS. The secondary purpose included the evaluation of differences in measurements of subpopulations between two consecutive visits (baseline and visit two) and evaluating the impact of T lymphocyte subset baseline values on PFS and OS.

2.5. Statistical analysis

Descriptive statistics, such as frequency (%) and median (Q1–Q3) were used to describe demographic, clinical, and laboratory

characteristics. Normality of the distribution was assessed using the Shapiro-Wilk test. Most of the investigated characteristics were non-normally distributed. Owing to non-normality, non-parametric statistical tests were used for statistical hypothesis testing. Fisher's exact test was used to assess differences in categorical demographic and clinical data between the treatment groups. Spearman correlation coefficients were used to estimate the relationship between age, CA 19–9, BMI, and counts of different lymphocyte subpopulations at the initial diagnosis in the study population. The Mann–Whitney *U* test and Kruskal–Wallis test were used to compare distributions between groups according to sex, presence of metastasis, and differentiation grade. The Wilcoxon Signed-Rank test was used to evaluate the level of CA 19–9 and counts of different lymphocyte subpopulations between baseline (visit one) and visit two.

Progression-free survival was defined as the interval between the first chemotherapy dose (baseline visit) and either disease progression or death. Overall survival was defined as the duration between the first chemotherapy dose (baseline visit) and death. The patients were censored at the last follow-up date if there were no PFS or OS events. Survival trends and median (95 % confidence interval [CI]) survival were analysed using the Kaplan–Meier method. Differences between survival curves were evaluated using log-rank and Breslow tests.

Univariate and multivariate Cox regression models were built to identify potential risk factors for PFS and OS. Hazard ratio (HR) and 95 % CI were calculated for each demographic, clinical, and laboratory variable. Factors found to be significant in the univariate Cox regression model were entered into the multivariate Cox model with the forward model selection process. Because there are no recognised cut-off values for inflammatory markers during survival analyses, lymphocyte subtypes were divided into two or four subgroups based on lower than median values or quartiles. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using IBM SPSS Statistics (version 23.0; IBM Corp., Armonk, NY, USA).

3. Results

3.1. Patient characteristics

A total of 106 patients were enrolled in the study. Fifty-six patients were treated with mFOLFIRINOX, and 46 patients were treated with gemcitabine monotherapy. Four patients could not be treated because of rapid deterioration. Baseline (visit one) samples were collected from 106 patients, and 46 patients' data from two visits were available: 25 patients from the FOLFIRINOX arm and 21 from the gemcitabine arm. The patient characteristics in both groups of chemotherapy regimens are depicted in Table 1. Only the ECOG status was significantly different between the groups.

Table 1
Summary of clinical characteristics of 102 patients treated with chemotherapy.

Characteristic	FOLFIRINOX (N = 56)	Gemcitabine (N = 46)	P value
Age, years			
Median	61.5	70.0	0.086
Range	43–78	55–84	
Sex, n (%)			
Female	37 (66.1)	22 (47.8)	0.104
Male	19 (33.9)	24 (52.2)	
Evidence of distant metastasis, n (%)			
M0	22 (39.3)	17 (37.0)	0.929
M1	34 (60.7)	29 (63.0)	
Ascites presented, n (%)			
Yes	52 (92.9)	6 (13.0)	0.191
No	4 (7.1)	40 (87.0)	
Tumour grade, n (%)			
G1	1 (1.8)	1 (2.2)	0.178
G2	33 (58.9)	21 (45.7)	
G3	22 (39.3)	24 (52.2)	
ECOG performance status score, n (%)			
0	37 (66.1)	18 (39.1)	0.004
1	17 (30.4)	20 (43.5)	
2	2 (3.6)	8 (17.4)	
Level of CA 19–9 (kU/L), n (%)			
$37 \leq$ CA 19–9 < 100	9 (16.1)	7 (15.2)	0.833
$100 \leq$ CA 19–9 < 1000	16 (28.6)	17 (37.0)	
$1000 \leq$ CA 19–9 < 12000	19 (33.9)	11 (23.9)	
$12000 \leq$ CA 19–9	12 (21.4)	11 (23.9)	
BMI (kg/m^2) category, n (%)			
BMI < 18.5	1 (1.8)	1 (2.2)	0.345
$18.5 \leq$ BMI < 25.0	27 (48.2)	23 (50.0)	
$25.0 \leq$ BMI < 30.0	13 (23.2)	17 (37.0)	
$30.0 \leq$ BMI < 35.0	12 (21.4)	4 (8.7)	
$35.0 \leq$ BMI	1 (1.8)	1 (2.2)	

3.2. Circulating lymphocyte subsets associated with clinicopathological characteristics

At the first visit, the association between individual demographic indicators and lymphocyte subpopulations was assessed. Correlation analysis revealed no relationship between the circulating subset counts and patient age. There were differences in total WBC (U [females, N = 60; males N = 45] = 825.50, z = -3.40, p = 0.001) and cytotoxic CD8 + CD57+ (U [females, N = 60; males, N = 45] = 1,033.00, z = -2.05, p = 0.04) counts according to sex. Total lymphocytes (U [M0, N = 40; M1, N = 65] = 863.50, z = -2.88, p = 0.004), B cells CD19+ (U [M0, N = 40; M1, N = 65] = 902.00, z = -2.63, p = 0.009), T helper CD3 + CD4+ (U [M0, N = 40; M1, N = 65] = 946.00, z = -2.34, p = 0.019) and CD3-CD56 + CD16- (U [M0, N = 40, M1, N = 65] = 981.00, z = -2.105, p = 0.035) cell counts differed between patients with and without distant metastases. Total WBC (H = 6.540, p = 0.038), T helper CD3 + CD4+ (H = 6.277, p = 0.043), and CD4 + CD25 + CD127+/- (H = 8.209, p = 0.017) counts correlated with tumour differentiation grade. A statistically significant positive correlation was observed between CA 19–9 level and CD8 + FOXP3 + count (r [100] = 0.239, p = 0.015). Body mass index was also positively correlated with NKT-like CD3 + CD56 + cells (r [100] = 0.21, p = 0.032) and cytotoxic CD8 + CD57 + T cells (r [100] = 0.23, p = 0.018).

3.3. Survival analysis

The median follow-up periods for all and alive patients were 27 months (range 0–54) and 29 months (range 13–54), respectively. Nine (8.5 %) patients survived at the last follow-up. Four (3.8 %) patients showed no disease progression. The median PFS was seven months (95 % CI: 6.2–7.8), and the median OS was 11.0 months (95 % CI: 8.7–13.3).

Progression-free survival differed and was 8.9 (95 % CI: 7.1–10.3) months in mFOLFIRINOX vs 7.1 (95 % CI: 4.8–9.3) months in the gemcitabine arm (p = 0.029, Breslow test); the HR for disease progression was 0.71 (95 % CI: 0.47–1.05, p = 0.089). The OS differed and was 15.1 (95 % CI: 12.3–18.0) months in mFOLFIRINOX vs 12.7 (95 % CI: 8.96–16.5) months in gemcitabine arm (p = 0.012, Breslow test); the HR for death was 0.79 (95 % CI: 0.52–1.92, p = 0.26).

In the univariate survival analysis for PFS, the factors with significant results ($p < 0.05$) included the presence of distant metastases, particularly in multiple sites, ECOG status, and the levels of absolute WBC counts, lymphocyte count, cytotoxic CD3 + CD8+, CD8 + CD57- cells, and NK CD3-CD56 + CD16- cells before treatment. (Supplementary Tables 1 and 2). However, we divided the patients according to chemotherapy arms, and the factors mentioned above remained significant in the FOLFIRINOX group, but the presence of distant metastases alone remained essential in the gemcitabine group (Supplementary Tables 3 and 4). Factors identified as significant during univariate analysis were studied using multivariate analysis. The presence of distant metastases, ECOG status, and CD8 + CD57- count before chemotherapy remained valuable for PFS.

In the univariate survival analysis for OS, age, ECOG status,

differentiation grade, CA 19–9, and the levels of absolute WBC counts, CD3 + CD8+, mainly CD8CD57- cells and CD4/CD8 ratio were significant (Supplementary Tables 5 and 6). Nevertheless, only the clinical factors remained significant when the chemotherapy regimen was considered (Supplementary Tables 7 and 8). In multivariate analysis for OS, independent prognostic factors included ECOG status, CA 19–9 level, and absolute WBC and CD8 + CD57- cell counts before treatment initiation.

Subsequently, we investigated the influence of individual subsets on survival using the Kaplan–Meier method, thereby distinguishing between two and four groups of each sub-population according to the median value or quartiles. After grouping CD8 + CD57- into two groups at the first visit (less and more than 194 cells/mL), we detected differences in PFS (Fig. 1A) and OS (Fig. 1B) in the whole study population and subgroups M0, M1, G2, G3, FOLFIRINOX, and gemcitabine arm.

We tried to understand the effect of chemotherapy on survival at different CD8 + CD57- values; perhaps one of the regimens is superior to the other in a particular group of patients. Twenty-five patients with a low CD8 + CD57- count (<194 cells/mL) and worse prognosis were analysed. Ten patients received FOLFIRINOX and 15 were treated with gemcitabine alone. Five patients from the gemcitabine arm survived for 8–24 months (age 64–78 years, four female, one male, two patients with G2 tumours, three patients with G3 tumours, and two patients with metastatic disease; their CA 19–9 varied between 241 kU/L and greater than 12000 kU/L. The ECOG status at diagnosis was 0–1, and the patients received 0–2 subsequent chemotherapy lines).

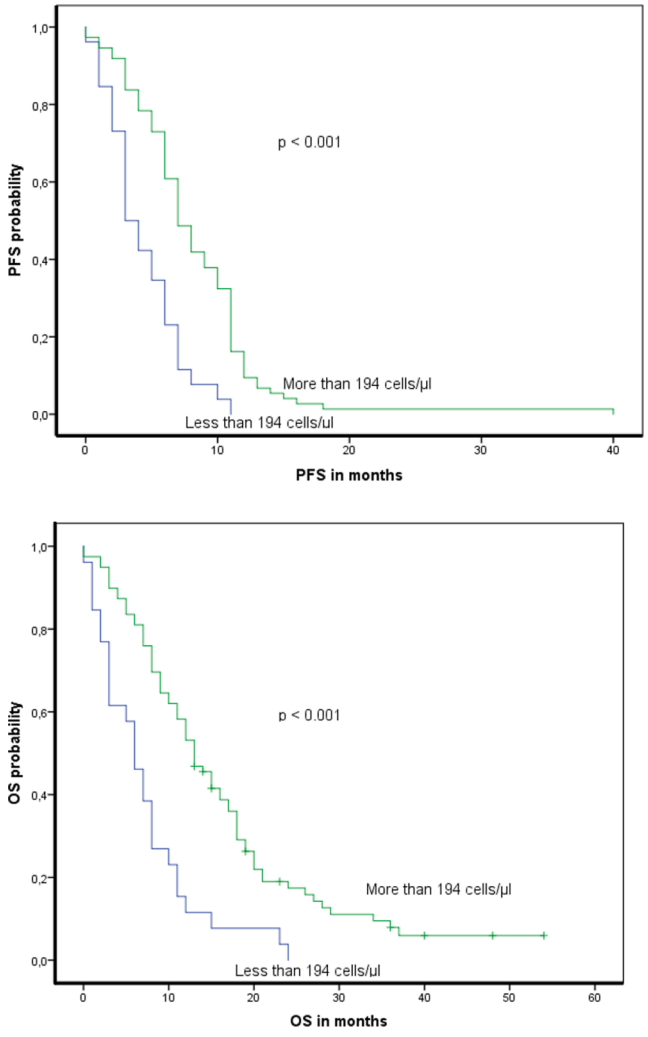

Fig. 1. Kaplan–Maier analysis of patients with different CD8CD57- cells according to a cut-off of 194 cells/µL. (A) PFS results. (B) OS results.

Absolute lymphocyte count at baseline demonstrated significant PFS and OS in the mFOLFIRINOX and gemcitabine arms, metastatic groups, and differentiation grade arms. CD4 + FOXP3 + cells also demonstrated significance in terms of OS; the cut-off was 49.57 cells/µL, and there was significant OS in differentiation grade 3, but not PFS, not in mFOLFIRINOX or gemcitabine, different metastatic statuses, and G2 tumours. CD3-CD56 + CD16- had an impact on PFS and was divided into two groups.

The means between the two visits were compared, and differences were confirmed for some subsets. However, these findings were not reliable for PFS and OS results. We subsequently examined differences in the frequencies of T cell subsets before treatment and after two months of chemotherapy (N = 46). We observed a decrease in the absolute numbers of NK CD3-CD56 + CD16 + and CD3-CD56 + CD16- and T regulatory CD4 + FOXP3 + cells and an increase in lymphocyte percentage and CD3 + CD56 + cells (Supplementary Tables 9). Significant differences in CD3 + CD4 + and CD3 + CD56 + cells were observed in the FOLFIRINOX arm and in CD8 + CD57- and CD4 + FOXP3 + cells in the gemcitabine arm. It appears that both regimens tended to affect CD3-CD56 + CD16 + counts (Table 2). Fig. 2 demonstrates how the CD8 + CD57- population counts changed between the two visits.

Interestingly NK CD3-CD56 + CD16- cells count at the second visit remained significant for PFS (HR, 0,994; 95 % CI: 0.987–1.000; p = 0.047) and not significant for OS (HR, 0.994; 95 % CI: 0.987–1.000; p = 0.051).

4. Discussion

In clinical studies, the mFOLFIRINOX regimen has revealed a clear advantage in PFS and OS in the metastatic setting [2]. No clear benefit for either regimen was seen in our study because patients were assigned not randomly, but according to physicians' choice based on patients' clinical status, mostly the ECOG performance status. However, despite this allocation, both chemotherapy regimen groups were fairly balanced and differed significantly only according to the ECOG performance status (Table 1).

Our analysis of lymphocyte subsets in advanced and metastatic PDAC treated with two different chemotherapy regimens demonstrated an association of CD3 + CD8 + T cells with PFS and OS. The CD8 + CD57- cells showed significance, whereas the CD8 + CD57 + subpopulation did not show such significance. Low values of CD8 + CD57- cells at the initial diagnosis before treatment led to a worse prognosis in the whole study population and in different subgroups. Our study demonstrated the value of circulating CD8 + CD57- as a prognostic factor, independent of disease spread (metastases) and aggressiveness (degree of differentiation). Due to the small number of patients, there is no reliable evidence that chemotherapy could influence the number of these cells and the prognosis of patients. However, we can say that different treatment regimens change the prognosis of patients with low initial CD8 + CD57-, and a low circulating CD8 + CD57- cell count is a predictive factor in modern chemotherapy.

Tumour infiltration by cytotoxic CD3 + CD8 + cells has long been considered a favourable prognostic factor for cancer patients, and recent studies have confirmed this [25–27]. Concerning PDAC, a *meta*-analysis of data showed the positive influence of CD3 + and significance of CD8 + cell localisation in the centre of the tumour [10]. The prognostic value of circulating CD3 + CD8 + subsets is questionable, but recently, in the era of new anti-cancer agents, especially immunotherapy, it is being studied more actively [28–31]. However, CD8 + T cells play an essential role in developing adaptive immunity against cancer [31].

CD8 + cytotoxic T cells are the leading players driving the adaptive immune response against cancer and they execute tumour-specific immune responses, rendering them the primary endpoint for most immunotherapies. Many facets of CD8 + *T*-cell dysfunction include tolerance, anergy, exhaustion, and senescence. Senescent T cells are characterised

Table 2
The means and significance of differences between means of the two visits for CA 19–9 and all lymphocyte subsets.

Parameters	FOLFIRINOX	N = 26		Gemcitabine	N = 21	
	Visit 1Mean (SD)	Visit 2Mean (SD)	*p* Value*	Visit 1Mean (SD)	Visit 2Mean (SD)	*p* Value*
CA 19–9 (kU/L)	3752.44	1623.43	**0.001**	2956.46	1586.10	**0.007**
	4524.33	3296.59		4580.90	3049.59	
WBC ($\times 10^9$/L)	7.50	6.28	**0.03**	7.8433	7.48	0.375
	2.38	2.79		2.58	5.30	
LYM ($\times 10^9$/L)	1.83	1.92	0.696	1.85	1.74	0.498
	0.59	0.68		0.72	0.59	
CD19+ (cells/μL)	233.55	189.72	0.192	165.69	168.76	0.414
	233.05	104.33		76.40	96.78	
CD3 + CD56+ (cells/μL)	118.04	156.89	**0.019**	114.38	124.22	0.092
	81.51	130.07		93.99	77.58	
CD8 + CD57+ (cells/μL)	122.27	133.82	0.563	123.89	137.08	0.230
	109.91	154.96		127.98	121.52	
CD8 + CD57- (cells/μL)	315.76	276.94	0.353	331.09	181.00	**0.014**
	140.74	144.91		228.92	103.42	
CD3 + CD8 + CD57+ (% out of CD8 +)	26.93	25.26	0.174	25.69	29.72	**0.027**
	17.92	16.79		19.10	16.95	
CD3 + CD4+ (cells/μL)	898.38	1051.76	**0.026**	867.90	908.95	0.958
	307.09	438.34		399.57	389.20	
CD3 + CD8+ (cells/μL)	425.29	483.95	0.078	446.36	398.05	0.543
	174.88	217.94		328.40	207.01	
CD3 + CD4-CD8- (cells/μL)	37.28	33.03	0.276	57.72	54.89	0.476
	30.90	21.81		39.58	41.50	
CD3-CD56 + CD16+ (cells/μL)	184.26	108.42	**0.005**	168.36	108.42	**0.004**
	142.92	57.43		148.28	104.31	
CD3-CD56 + CD16- (cells/μL)	58.59	48.12		76.05	41.20	**0.004**
	79.63	69.56		80.20	43.64	
CD4 + CD25 + CD127+/- (cells/μL)	57.25	49.07	0.069	62.54	58.89	0.476
	25.87	26.01		30.52	25.82	
CD4 + FOXP3+ (cells/μL)	36.21	31.62	0.158	37.58	24.50	**0.046**
	21.28	16.47		28.79	14.23	
CD8 + CD25 + CD127+/- (cells/μL)	1.02	1.230	0.976	0.72	0.43	0.380
	1.86	2.33		1.18	0.30	
CD8 + FOXP3+ (cells/μL)	0.42	0.68	0.578	0.34	0.16	0.472
	0.51	1.12		0.80	0.16	
CD4/CD8	2.29	2.31	0.861	2.89	2.93	0.274
	0.77	0.79		2.04	2.05	

* Differences in means between the two visits.
CA 19–9 – carcinoma antigen CA 19–9, WBC – white blood cell, LYM – lymphocytes, SD – standard deviation.

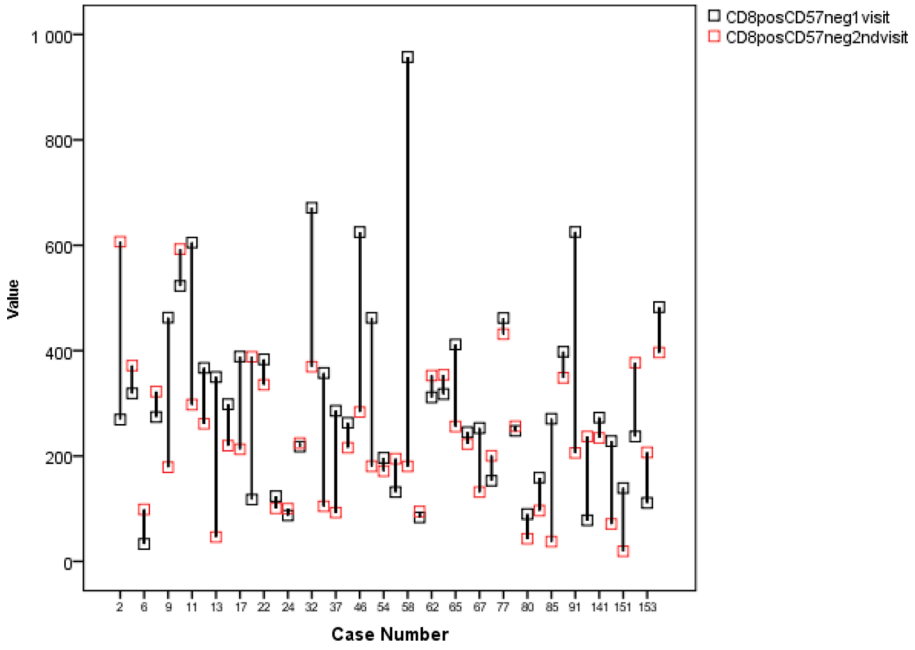

Fig. 2. CD8 + CD57- population counts changed between the two visits in some individual patients.

by lowered expression of CD28 and increased expression of the CD57 antigen [32]. Several reports have indicated that the levels of CD8 + CD28- or CD8 + CD57 + T cell subsets are increased in both peripheral blood and TME of patients with solid tumours and haemato-oncological malignancies [33]. We failed to demonstrate a negative relationship between the levels of CD8 + CD57 + T lymphocytes and survival of PDAC patients. However, the prognostic significance of peripheral blood CD8 + CD57- T lymphocytes supports the view that nonsenescent T lymphocytes are crucial for tumour control in PDAC; thus, blocking T lymphocyte senescence to enhance immune tumour control in PDAC seems promising [32]. In the gemcitabine monotherapy and FOLFIRINOX arms, CD3 + CD8 + and CD8 + CD57- levels demonstrated prognostic significance. In contrast, patients with low initial values had better OS results than those treated with gemcitabine alone.

The effects of gemcitabine on CD3 + CD8 + cells have been studied *in vitro* [34], but there is a lack of data on its influence on immunostimulation *in vivo*. Furthermore, the immunostimulatory or inhibitory value of mFOLFIRONOX has not been extensively studied. However, neoadjuvant FOLFIRINOX studies have revealed the ability of this combination to induce CD8 + cell infiltration [20,35]. Our results suggest that gemcitabine may better stimulate an increase in CD8 + CD57- counts, thereby improving patient survival.

The absolute lymphocyte count may also be considered an essential prognostic indicator in human cancer and PDAC, but the data are heterogeneous [35]. Our data confirmed that patients with lower absolute lymphocyte values measured by flow cytometry demonstrated poorer survival results in different groups, suggesting that they could be qualified as a prognostic factor. Nevertheless, one should consider which subpopulation determines the prognostic value.

The baseline T regulatory CD4 + FOXP3 + value was prognostic, but only in the general population, G3 group, and exceptionally for OS. This finding led to the conclusion that the number of these cells in the blood is related to worse tumour differentiation and is not an independent predisposing factor.

Infiltration by CD4 + FOXP3 + cells in the TME is usually considered a poor prognostic factor for different cancer types [36,37], including PDAC [38]. There is little data regarding circulating CD4 + FOXP3 in the PDAC population. However, their lower amount is also associated with a better prognosis [39]. Gemcitabine chemotherapy seems to reduce circulating CD4 + CD25 + FOXP3 + in non-small cell lung cancer [40], and the FOLFIRINOX regimen induces some changes as well [23]. The same authors showed no impact of changes in circulating Tregs in PDAC on survival after 30 days of chemotherapy initiation. According to our results, chemotherapy-induced changes mainly decreased the number of circulating T regulatory CD4 + FOXP3 cells. However, there was no impact of this effect on PFS or OS when analysing both chemotherapy regimens and the gemcitabine and FOLFIRINOX groups.

In our study, lower NK CD3-CD56 + CD16- levels at baseline correlated with poor PFS but not OS, and in specific groups according to stage, differentiation grade, and chemotherapy regimens, no effect on survival was confirmed. Changes in these cells and their importance in solid tumours have not been widely described. Gemcitabine appears to increase its activation in mouse models of lung cancer. The same analysis revealed changes between visits but no impact on survival results [41].

Most previous studies were based on tumour-infiltrating lymphocytes, retrospective, and had small sample sizes or a single outcome variable. Our study aimed to determine whether the state of the immune system influences the effectiveness of chemotherapy and whether chemotherapy can change the immune system. We assessed this by observing changes in the peripheral blood. It is worth noting that although variations in peripheral blood are easily monitored by routine blood sampling, they may not fully reflect the situation within the tumour. Additionally, these fast and low-cost blood biomarkers are used to identify the patient's immune status, but the impact of chemotherapy or other systemic treatments on circulating biomarkers of the immune system requires further research. We did not observe substantial differences in lymphocyte subtypes among most clinical groups and no significant changes during treatment, suggesting that some lymphocyte subsets (mainly CD8 + CD57- cells) are promising independent predictive biomarkers.

Our study results suggest that lymphocyte subtypes before chemotherapy predict prognosis, but changes during treatment are not significant for PFS and OS. Our study revealed the influence of circulating T cells on survival in patients with advanced and metastatic pancreatic cancer treated with conventional chemotherapy. This refutes the hypothesis that cell changes during treatment affect survival. Additionally, this is the only study comparing two groups of patients treated with different chemotherapy regimens, and one of the few to examine the effect of the FOLFIRINOX regimen on the immune system.

Our study limitations include small and heterogeneous cohort and short intervals between blood sample testing. Patients who fell into one or another chemotherapy group were not randomised according to functional status or age; as such, randomisation would further reduce the number of groups and statistical reliability. However, immune profile may differ with age. When analysing the differences in age groups in our study, no reliable distinctions were found between the chemotherapy groups. Longer intervals between visits could also better reveal the long-term effects on immune cells. It was slightly disappointing to find that differences induced by chemotherapeutic agents did not influence the survival results. These negative results could be due to the small sample size and suggest room for improvement in ongoing studies. Additionally, future studies could include a wider spectrum of immune cells, and it would be interesting to compare the data of circulating cells and factors of the TME to study the effect of new chemotherapy drugs, such as nab-paclitaxel, liposomal pegylated irinotecan, targeted agents, or immunotherapy on circulating lymphocytes.

In conclusion, as precision medicine and Immuno-oncology in PDAC mature, this study looks forward to innovative biomarkers and personalized cancer care.

5. Ethics approval and consent to participate

This study was approved by the Vilnius Regional Bioethics Committee, and written informed consent was obtained from all patients prior to the study.

6. Consent for publication

All authors read and approved the final manuscript for publication.

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Skaiste Tulyte: Conceptualization, Methodology, Software, Validation, Investigation, Writing – original draft, Writing – review & editing. **Dainius Characiejus:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Reda Matuzeviciene:** Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Ausra Janiulioniene:** Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Mantas Radzevicius:** Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. **Elena Jasiunaite:** Resources, Data curation, Writing – original draft, Writing – review & editing. **Tadas Zvirblis:** Software, Validation, Writing – original draft, Writing – review & editing. **Audrius Sileikis:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2023.109722.

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